Role of *Scn1b* in Colonic Myenteric Neuron Function in a Model of Developmental and Epileptic Encephalopathy

by

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Dedication

To my dedicated colleagues
and my inspiring friends
and my supportive parents, Susan and Gary
and my precious cats, Lucy and Brian
and my beloved partner, Vinodh

Truly, I would not have reached this point without all of you.

Acknowledgements

This work took a village (or – perhaps – an entire city). I want to acknowledge my ever-supportive PI and lab mates, insightful committee members, and generous collaborators (across multiple institutions and states) for their invaluable roles in generating this body of work. I am honored that you all chose to be part of my scientific journey – and I am more grateful than I could ever fully or adequately express.

Thank you.

Table of Contents

List of Tables

List of Figures

Abstract

Many neurological diseases present with comorbid gastrointestinal (GI) abnormalities, suggesting parallel altered functionality of the central nervous system (CNS) and enteric nervous system (ENS). A potential mechanistic link between CNS and GI disorders is aberrant function of voltage-gated sodium channel (VGSC) α and β subunits, which are expressed in both CNS and ENS. VGSCs control the generation and propagation of action potentials in neurons and therefore play a critical role in neuronal excitability and signaling. VGSC β1/β1B subunits, encoded by *SCN1B*, modulate ion channel expression, trafficking, gating, and voltage-dependence. VGSC gene variants are linked to developmental and epileptic encephalopathies (DEEs) and GI motility disorders. Additionally, DEE patients with VGSC – and other voltage-gated ion channel (VGIC) gene variants – often exhibit chronic constipation. The overall goal of my PhD thesis research was to test the hypothesis that aberrant activity or expression of VGICs contributes to the mechanism of GI dysfunction in the *Scn1b*-null mouse model of DEE. The first research project contained in this thesis investigated GI symptoms among patients with DEEs linked to variants in VGIC genes in an effort to identify commonalities between patients exhibiting GI symptoms. We found that GI symptoms are prevalent and often severe in DEE patients and are not fully explained by factors often implicated in GI dysfunction, e.g. functional mobility, diet, or medications. By focusing on DEE patients with variants in genes encoding VGICs, these results highlight the need to better understand the role of

aberrant function of voltage-gated ion channels that may directly contribute to the observed GI pathology.

The second portion of my thesis research investigated a functional role of VGSC β1/β1B subunits in GI motility. A subset of DEE patients has biallelic loss-of-function variants in *SCN1B*. In addition to aberrations in neuronal firing within the CNS, multiple physicians report *SCN1B*-linked DEE patients presenting with GI symptomology, including constipation. The objective of this project was to understand how β1/β1B subunits contribute to GI function using a *Scn1b*-null mouse model. Our results indicate that β1/β1B are involved in maintaining proper frequency and regularity of colonic contractions, likely via transcriptional regulation of voltage-gated ion channel expression.

Taken together, the results of my thesis work shed light on the prevalence and potential causes of GI symptoms in VGIC-linked diseases, including DEE, and provide a mechanistic role for *SCN1B* in colonic motility.

Chapter 1 : Introduction

1.1 A Proposed Mechanism for the Gut-Brain Disease Connection

There is a growing body of evidence suggesting a significant connection between central nervous system (CNS) and gastrointestinal (GI) dysfunction. Specifically, clinical studies have reported high incidences of bowel dysmotility in patients with multiple forms of epilepsy as well as neurological epilepsy comorbidities, such as autism spectrum disorders, depression, anxiety, and multiple sclerosis (Bassotti et al., 1998; Gershon, 2005; Gorard, Gomborone, Libby, & Farthing, 1996; Mazurek et al., 2013; Nordenbo, Andersen, & Andersen, 1996; Norton & Chelvanayagam, 2010; M. Rao & Gershon, 2016; Winge, Rasmussen, & Werdelin, 2003). Likewise, neuronal mechanisms have been implicated in GI diseases often characterized by constipation, such as irritable bowel syndrome (IBS), which is thought to be partially caused by altered activation of enteric neurons, and Hirschsprung's disease (HSCR), which is caused by an absence of neurons in distal bowel (Gershon, 2005; O'Donnell, Coyle, & Puri, 2016). The large degree of overlap between CNS and GI disorders suggests one or more mechanistic links between these two physiological systems in disease states. One potential explanation for co-manifestation of CNS and GI symptoms is a shared cause of neuronal dysfunction in these two organ systems, and the goal of my thesis work was to investigate one such candidate mechanism: aberrant voltage-gated sodium channel (VGSC) function.

VGSC-associated gene variants are linked to multiple neurological diseases, including developmental and epileptic encephalopathies (DEEs) (Aeby et al., 2019; Chahine, Chatelier, Babich, & Krupp, 2008; Kaplan, Isom, & Petrou, 2016; O'Malley & Isom, 2015). Notably, chronic or severe constipation is reported in multiple case studies of DEE patients with variants in VGSC and other voltage-gated ion channel (VGIC)-encoding genes (Table 1.1) as well as patients with variants in *FHF1*, which encodes a fibroblast-growth-factor homologous factor that is known to modulate VGSC inactivation (Al-Mehmadi et al., 2016; Berecki et al., 2018; de Kovel et al., 2014; Leipold et al., 2013). Furthermore, variants in the VGSC-encoding genes *SCN5A* and *SCN11A* have been linked to GI pathologies, such as IBS and HSCR (Neshatian et al., 2015; O'Donnell et al., 2016; Saito et al., 2009; Strege et al., 2018; Woods, Babiker, Horrocks, Tolmie, & Kurth, 2015). These clinical findings show that variants impacting VGSC function often produce both neurological and GI symptomology.

The studies contained in this thesis investigate the role of VGSCs – and specifically their β subunits – in GI function and dysfunction. Chapter 1 provides a background on the established physiological functions of VGSCs and their β subunits, the organization of the enteric nervous system, the different patterns of GI motility, and the previously known roles of VGSCs in the GI tract. Chapter 2 focuses on the clinical presentation of GI symptoms in patients with channelopathy DEEs and explores potential causative factors in this population. Chapter 3 presents experiments that interrogate the role of VGSC β subunits in enteric nervous system development and function in a mouse model of

channelopathy DEE. Chapter 4 discusses future avenues of research that may reveal additional functions of VGSC β subunits in GI and gut-brain-axis function. Overall, these findings suggest that VGSC β subunits are critical for the coordinated signaling of enteric neurons and proper functioning of the GI tract.

Table 1.1: Gastrointestinal symptoms in VGIC-linked channelopathies

ION CHANNEL GENE (PROTEIN PRODUCT)	DISEASE(S) ASSOCIATED WITH GENETIC VARIANT THAT MANIFEST WITH GI SYMPTOMS	KNOWN LOCALIZATION OF GENE AND/OR PROTEIN PRODUCT IN HUMAN GI TRACT	KNOWN GI SYMPTOMS ASSOCIATED WITH GENETIC VARIANT
***SCN1A* ($NA_V1.1$)**	OMIM DEE6 (Claes et al., 2001; Dravet, 2011)	Colonic MP, colonic mechanosensitive nerve fibers (Hetz et al., 2014; Osteen et al., 2016)	Appetite disturbances, constipation, intestinal dysmotility (Beck, Isom, & Berg, 2021; Villas, Meskis, & Goodliffe, 2017; Ziobro, Eschbach, Sullivan, & Knupp, 2018)
***SCN1B* (NA_V β1/B SUBUNITS)**	OMIM DEE52 (Aeby et al., 2019)	Colonic ICCs, colonic PDGFRα^+ cells, colonic MP, colonic SP, colonic smooth muscle (O'Donnell, Nakamura, Tomuschat, Marayati, & Puri, 2019)	Constipation, FTT (Personal_Communication, 2019 & 2020)
***SCN2A* ($NA_V1.2$)**	OMIM DEE11 (Berecki et al., 2018; Howell et al., 2015)	Colonic MP, colonic smooth muscle (Hetz et al., 2014)	Constipation, diarrhea, FTT, vomiting (Berecki et al., 2018; Howell et al., 2015)
***SCN3A* ($NA_V1.3$)**	OMIM DEE62 (Zaman et al., 2018)	Colonic MP, colonic smooth muscle, enterochromaffin cells in small intestine and colon (Hetz et al., 2014; Strege et al., 2017)	FTT (Zaman et al., 2018)
***SCN4A* ($NA_V1.4$)**	NDM (Gay et al., 2008)	Colonic smooth muscle (Hetz et al., 2014)	Constipation, FTT, gastroesophageal reflux (Gay et al., 2008)
***SCN5A* ($NA_V1.5$)**	IBS, LQTS (Beyder et al., 2014; Locke, Ackerman, Zinsmeister, Thapa, & Farrugia, 2006; Saito et al., 2009; Strege et al., 2018)	Colonic MP, ICCs in small intestine, smooth muscle in small intestine and colon (Hetz et al., 2014; Holm et al., 2002; Neshatian et al., 2015; Ou et al., 2002; Strege, Holm, et al., 2003; Strege, Ou, et al., 2003)	Abdominal pain, constipation, diarrhea (Beyder et al., 2014; Locke et al., 2006; Saito et al., 2009; Strege et al., 2018)
***SCN8A* ($NA_V1.6$)**	OMIM DEE13 (Gardella & Møller, 2019)	Colonic MP (Hetz et al., 2014)	Constipation, gastroesophageal reflux (Gardella & Møller, 2019)
***SCN9A* ($NA_V1.7$)**	NPS/SFN (Cannon, Kurklinsky, Guthrie, & Riegert-Johnson, 2016; Eijkenboom et al., 2019; Hisama,	Colonic MP, colonic smooth muscle (Hetz et al., 2014)	Abdominal/rectal pain, constipation, diarrhea, (Cannon et al., 2016; Eijkenboom et al., 2019; Hisama et al., 1993)

	Dib-Hajj, & Waxman, 1993)		
***SCN10A* ($NA_V1.8$)**	NPS/SFN (Dabby et al., 2016; Eijkenboom et al., 2019)	Colonic MP (Hetz et al., 2014)	Abdominal discomfort, constipation, diarrhea, gastroesophageal reflux, gastroparesis, vomiting (Dabby et al., 2016; Eijkenboom et al., 2019)
***SCN11A* ($NA_V1.9$)**	CIP, NPS/SFN (Eijkenboom et al., 2019; Leipold et al., 2013; Woods et al., 2015)	Colonic MP, colonic SP, colonic smooth muscle (Hetz et al., 2014; O'Donnell et al., 2016)	Abdominal/perineal/rectal pain, constipation, diarrhea, enlarged colon, FTT, intestinal dysmotility (Eijkenboom et al., 2019; Leipold et al., 2013; Woods et al., 2015)
***KCNB1* ($K_V2.1$)**	OMIM DEE26 (Marini et al., 2017; Srivastava et al., 2018)	Unknown in humans; colonic MP in mice (Seifi et al., 2014)	Constipation, FTT, intestinal dysmotility (Beck et al., 2021; Marini et al., 2017)
***KCNQ2* ($K_V7.2$)**	OMIM DEE7 (Lee, Chang, Liang, & Li, 2019)	Unknown; K_V7 channels localized to colonic sensory afferents in humans and colonic MP in mice (Jepps, Greenwood, Moffatt, Sanders, & Ohya, 2009; Peiris et al., 2017)	Constipation, intestinal dysmotility (Beck et al., 2021)
	Abbreviations: CIP = congenital insensitivity to pain; DEE = developmental and epileptic encephalopathy; FTT = failure-to-thrive; IBS = irritable bowel syndrome; ICCs = interstitial cells of Cajal; LQTS = congenital long QT syndrome; MP = myenteric plexus; NDM = non-dystrophic myotonia; NPS = neuropathic pain syndromes; PDGFRα^+ = platelet-derived growth factor receptor α^+; SFN = small fiber neuropathy; SP = submucosal plexus		

1.2 The Function of Voltage-Gated Sodium Channels and their β Subunits

Voltage-gated sodium channels (VGSCs)

VGSCs are responsible for the initiation and propagation of action potentials (APs) in central and peripheral neurons, GI pacemaker cells, smooth and skeletal muscle cells, and cardiac myocytes – though they are also present in non-excitable cells and tissues, such as astrocytes, glia, cardiac fibroblasts, breast cancer cells, prostate cancer cells, and intestinal epithelial cells (Aronica et al., 2003; Barshack et al., 2008; Beyder & Farrugia, 2012; Brackenbury & Isom, 2011; Brunklaus, Ellis, Reavey, Semsarian, & Zuberi, 2014; Catterall, 2000; Fein, Wright, Slat, Ribera, & Isom, 2008; Maier et al., 2004; O'Malley & Isom, 2015; Oh, Lee, & Waxman, 1997). In neurons, VGSCs are localized at high density in the axon initial segment, where the AP is initiated, and – in myelinated neurons – in nodes of Ranvier, where APs are regenerated and thereby propagated along the axon during saltatory conduction (Kole et al., 2008; Komada & Soriano, 2002). VGSCs are thought to be important for the normal excitability of interstitial cells of Cajal (ICCs), which integrate enteric motor neuron input to direct GI smooth muscle relaxation and contraction (Strege, Ou, et al., 2003); likewise, VGSCs are also known to be concentrated at neuromuscular junctions, where they conduct sodium ions and thus initiate APs in skeletal muscle fibers (Simkin & Bendahhou, 2011). In cardiomyocytes, VGSCs are primarily clustered at the intercalated disks and t-tubules, where they are vital for normal conduction velocity and excitation-contraction coupling (Dhar Malhotra et al., 2001; Haufe et al., 2005; Kaufmann et al., 2013; Maier et al., 2004).

Each VGSC is a heterotrimeric protein complex composed of one pore-forming α subunit and two distinct β subunits: either a β1 or β3, which non-covalently associate with the α subunit, and a β2 or β4, which covalently associate with the α subunit via disulfide bonds (Catterall, 2000; Isom et al., 1992; Morgan et al., 2000; O'Malley & Isom, 2015; Yu et al., 2003). There are nine identified VGSC α subunits, $Na_v1.1$-$Na_v1.9$, which can be categorized by their relative sensitivity to tetrodotoxin (TTX) (Table 1.2) (Catterall, Goldin, & Waxman, 2005; O'Malley & Isom, 2015). TTX-sensitive (TTX-S) VGSC α subunits can be blocked by nanomolar concentrations of TTX, while TTX-resistant (TTX-R) VGSC α subunits require micromolar concentrations of TTX to impair function (Catterall et al., 2005; O'Malley & Isom, 2015). VGSC β subunits regulate channel activity and cell-surface expression in addition to functioning as cell adhesion molecules (CAMs) and transcriptional modulators (Aeby et al., 2019; Bouza et al., 2021; Calhoun & Isom, 2014; Isom, 2001, 2002; Isom & Catterall, 1996). There are five known VGSC β subunits encoded by four genes (Table 1.2) (O'Malley & Isom, 2015). The genes encoding these β subunits are involved in myriad physiological processes throughout the body, and β subunit gene variants have been implicated in pathologies such as ataxia, autism, cancer, cardiac arrhythmia, epilepsy, neurodegeneration, neuropathic pain, and sudden unexpected death in epilepsy (SUDEP) (Aeby et al., 2019; Calhoun & Isom, 2014; O'Malley & Isom, 2015).

Table 1.2: VGSC genes, encoded proteins, and relevant properties

α SUBUNIT GENE	α SUBUNIT PROTEIN	TTX-SENSITIVITY	β SUBUNIT GENE	β SUBUNIT PROTEIN(S)
SCN1A	$Na_v1.1$	TTX-S	***SCN1B***	β1, β1B
SCN2A	$Na_v1.2$	TTX-S	***SCN2B***	β2
SCN3A	$Na_v1.3$	TTX-S	***SCN3B***	β3
SCN4A	$Na_v1.4$	TTX-S	***SCN4B***	β4
SCN5A	$Na_v1.5$	TTX-R		
SCN8A	$Na_v1.6$	TTX-S		
SCN9A	$Na_v1.7$	TTX-S		
SCN10A	$Na_v1.8$	TTX-R		
SCN11A	$Na_v1.9$	TTX-R		

VGSC β1/β1B subunits

This thesis focuses on the physiological roles of VGSC β1/β1B subunits. *SCN1B* encodes the VGSC β1 subunit and its soluble splice variant, β1B, which play roles in cell signaling and modulate both sodium and potassium currents (Aeby et al., 2019; Brackenbury & Isom, 2011; Davis, Chen, & Isom, 2004; Hull et al., 2020; Lopez-Santiago, Brackenbury, Chen, & Isom, 2011; Lopez-Santiago et al., 2007; O'Malley & Isom, 2015; Patino et al., 2009; Patino & Isom, 2010; Yuan et al., 2019). β1 is a transmembrane protein that associates with VGSC α subunits, potassium channel α subunits, CAMs, extracellular matrix proteins, and cytoskeletal proteins (Brackenbury et al., 2010; Brackenbury & Isom, 2011; Calhoun & Isom, 2014; Marionneau et al., 2012; McEwen & Isom, 2004). β1B is a developmentally regulated, secreted *SCN1B* isoform that modulates Nav1.5-mediated sodium current (I_{Na}) – but not I_{Na} generated by TTX-sensitive VGSCs – and functions as a soluble ligand for cell adhesion (Patino et al., 2011). β1 and β1B exhibit inverse

expression patterns in the developing rodent brain; β1B is primarily expressed during embryonic development and early life, while β1 expression becomes detectable during postnatal development and peaks during adulthood (Isom et al., 1992; Kazen-Gillespie et al., 2000; Patino et al., 2011). Conversely, β1B expression remains high in the rodent cardiac system into adulthood (Edokobi & Isom, 2018; Kazen-Gillespie et al., 2000).

Importantly, variants in *SCN1B* are linked to disease. A subset of DEE patients has homozygous recessive loss-of-function (LOF) variants in *SCN1B*, and physicians report that these *SCN1B*-linked DEE (DEE52) patients exhibit intractable seizures, developmental delay, ataxia, and increased risk for SUDEP (Aeby et al., 2019; Ogiwara et al., 2012; Patino et al., 2009; Personal_Communication, 2019 & 2020; Ramadan et al., 2017). DEE52 patients are also reported to exhibit GI symptoms, including constipation (Personal_Communication, 2019 & 2020). Additionally, a recent clinical study showed reduced *SCN1B* mRNA expression in both ganglionic and aganglionic portions of colon in HSCR patients (O'Donnell et al., 2019).

VGSC β subunits were traditionally thought to be accessory proteins that functioned to modulate the gating and kinetics of VGSCs. Foundational experiments in Xenopus oocytes showed that co-expression of β1 with $Na_v1.2$ accelerates the channel's inactivation, shifts the voltage dependence of inactivation to more negative membrane potentials, and increases the size of the peak sodium current (Isom et al., 1992). When expressed in immortalized mammalian cell lines, β1 was similarly observed to increase peak sodium current density and induce a negative shift in the voltage dependence of

inactivation yet had no effect on the rate of inactivation in these systems (Aeby et al., 2019; McEwen, Meadows, Chen, Thyagarajan, & Isom, 2004; Meadows et al., 2002; Patino et al., 2009). Additionally, experiments performed in mammalian cell lines show that β1/β1B subunits influence α subunit surface expression; however, the observed impact of β1/β1B subunits differed based on the cell line used in a given study (Calhoun & Isom, 2014; Kazarinova-Noyes et al., 2001; Kazen-Gillespie et al., 2000; McEwen et al., 2004).

Studies performed in heterologous or other *in vitro* systems established key projections for the context-dependent, physiological roles of β1 subunits *in vivo*. *Scn1b*-null mice, which lack β1 and β1B expression, exhibit a phenotype which mirrors the symptoms of DEE52 patients – including spontaneous and generalized seizures, failure-to-thrive, ataxia, and premature death around post-natal day (P) 19 (Chen et al., 2004; Yuan et al., 2019). Mouse studies have also reinforced the canonical roles of VGSC β1 subunits: modulating the cell-surface expression and activity of the VGSC α subunits. Recent work in the Isom lab suggests that the cleaved intracellular domain of β1 subunits can translocate to the nucleus and regulate gene transcription of VGSCs as well as other voltage-gated ion channels (VGICs) (Bouza et al., 2021). The *Scn1b*-null hippocampal CA3 region has decreased $Na_v1.1$ expression and increased $Na_v1.3$ expression compared to aged-matched wild-type (WT) mice (Chen et al., 2004). *Scn1b*-null cardiac myocytes also exhibit increased $Na_v1.3$ levels in addition to increased $Na_v1.5$ expression, and dorsal root ganglion (DRG) neurons exhibit decreased cell-surface $Na_v1.9$ expression (Brackenbury et al., 2010; Lin et al., 2015; Lopez-Santiago et al., 2011; Lopez-Santiago

et al., 2007). These studies show that VGSC β1 subunits impact VGSC α subunit expression levels in the CNS, peripheral nervous system (PNS), which consists of the nerves and ganglia located outside of the brain and spinal cord, and cardiac system in a cell-type-specific manner.

Scn1b deletion results in cell-type-specific effects on excitability in the CNS, PNS, and cardiac systems. Previous work suggests *Scn1b*-null cerebellar neurons are hypoexcitable, while *Scn1b*-null cortical pyramidal neurons exhibit a biphasic response in which they are hyperexcitable at lower injected currents and hyperexcitable at higher injected currents (Hull et al., 2020; Reid et al., 2014). In addition to VGSCs, β1 subunits influence activity of voltage-gated potassium channels (VGKCs); co-immunoprecipitation experiments suggest that β1 and K_v4.2 interact in mouse brain, and knockdown of β1 in cultured cortical neurons reduced potassium current, likely due to decreased cell-surface expression of VGKCs (Kearney, 2013; Marionneau et al., 2012). Furthermore, nociceptive *Scn1b*-null DRG neurons are hyperexcitable, due to modulations in both sodium and potassium currents (I_{Na} and I_K, respectively) (Lopez-Santiago et al., 2011). These data show that *Scn1b* plays an important role in maintaining normal excitability in neurons and other excitable cells throughout the body.

As well as playing an integral role in channel modulation, β1 subunits are members of the immunoglobulin superfamily of cell adhesion molecules (CAMs), which regulate cell adhesion, proliferation, and migration (Brackenbury & Isom, 2011; Calhoun & Isom, 2014). Furthermore, β1 subunits have been shown to modulate axon outgrowth and

pathfinding (Brackenbury et al., 2010; Brackenbury et al., 2008; Calhoun & Isom, 2014; Davis et al., 2004). These additional functions of β1 subunits suggest their importance in normal nervous system development. *Scn1b*-null mice have significantly fewer nodes of Ranvier in optic nerve, aberrations in axo-glial junctions in CNS and PNS axons, and reduced myelination of sciatic nerve and spinal cord (Chen et al., 2004). Previous studies show that both the extracellular domain of β1 and its soluble splice variant, β1B, promote neurite outgrowth in cerebellar granule neurons via CAM-mediated signaling (Davis et al., 2004; McEwen & Isom, 2004; Patino et al., 2011). These small neurite extensions protruding from immature neurons establish polarity, develop into axons and dendrites, and begin to form the connections that eventually define a neural circuit. In the CNS, it has been shown that β1/β1B subunits mediate axon fasciculation, the process by which growing axons adhere to one another to limit the dispersion of axonal tracks and generate more precise connections between regions (Brackenbury, Yuan, O'Malley, Parent, & Isom, 2013). *Scn1b*-C121W mice, a mouse model based on a human *SCN1B* variant, exhibit reduced dendritic arborization in the pyramidal neurons of the somatosensory cortex (Reid et al., 2014). *Scn1b*-null mice, which lack β1/β1B-mediated neuronal outgrowth, exhibit disrupted axonal pathfinding and fasciculation in the cerebellum as well as in the corticospinal tract (Brackenbury et al., 2008). These data show that VGSC β1 subunits are vital for the formation of proper neuronal networks.

In conclusion, the vital roles that VGSC β1 subunits play in mammalian neuronal excitability and development have been extensively studied and documented in the brain and spinal cord; however, CNS neurons are not the only neurons in the body known to express VGSCs. The largest population of neurons located outside the CNS is the enteric

nervous system situated in the GI tract, and the potential function(s) of β1 subunits in these neurons has not yet been established.

1.3 The Gastrointestinal Tract and Enteric Nervous System Function

The digestive system and gastrointestinal tract

The overall function of the digestive system is to is to chemically and mechanically break down ingested material, absorb the resulting nutrient molecules, and then excrete the remaining fecal material (Ogobuiro, Gonzales, & Tuma, 2021). The key digestive organs – in order of food processing – are the oral cavity, pharynx, esophagus, stomach, small intestine (also referred to as the small bowel), and large intestine (also referred to as the large bowel or colon). This section will focus primarily on the roles of the small and large intestines.

The vast majority (90%) of food molecule and nutrient absorption occurs in the small intestine (Ogobuiro et al., 2021). The small intestine is segmented into three portions: the most “proximal” portion - duodenum, which receives contents from the stomach, jejunum, and the most “distal” portion - ileum, which releases contents to the large intestine (Fish & Burns, 2021; Ogobuiro et al., 2021). The small intestine produces and secretes a large amount of hormones and enzymes in response to the specific components of the materials passing through, enabling it to digest and absorb carbohydrates, proteins, lipids, vitamins, and minerals (Fish & Burns, 2021). The epithelial layer of the small intestine, which borders the intestinal lumen through which the digested materials flow, is highly convoluted; these villi and microvilli increase the surface area available for absorption of nutrients (Fish & Burns, 2021; Ogobuiro et al., 2021). After passing through the small intestine, digested material moves to the colon. The colon is primarily responsible for

absorbing water and electrolytes and then finally excreting the fecal material from the body through the rectum (Debongnie & Phillips, 1978; Ogobuiro et al., 2021; Phillips & Giller, 1973). In the small and large bowels, coordinated contractions and relaxations move material to the next location while mixing the ingested food with digestive enzymes and intestinal secretions, thus enabling nutrient breakdown into absorbable materials as well as exposing these materials to the lumen for subsequent absorption; all of these activities are primarily controlled by localized neuronal networks known as the enteric nervous system (ENS) (Hansen, 2003; Huizinga et al., 2011).

The organization and function of the enteric nervous system

The peripheral nervous system (PNS) composes the nerves and ganglia that are located outside the brain and spinal cord; a component of the PNS is the autonomic nervous system (ANS), which regulates involuntary physiological functions (Waxenbaum, Reddy, & Varacallo, 2021). The largest branch of the ANS is the ENS, an extensive network of over 100 million neurons embedded in the walls of the intestinal tract (M. Rao & Gershon, 2016; Waxenbaum et al., 2021). The ENS is capable of regulating GI functions independently of input from brain or spinal cord; this ability is particularly apparent in the small and large intestines, which retain normal motility patterns in the complete absence of CNS innervation (M. Rao & Gershon, 2016; Waxenbaum et al., 2021).

The ENS is composed of two neuronal plexuses: the submucosal plexus and the myenteric plexus. The submucosal plexus is located only in the small and large bowel, while the myenteric plexus extends the entire length of the GI tract (Furness, Callaghan,

Rivera, & Cho, 2014). Previous studies have shown that the myenteric plexus develops before the submucosal plexus, though both are present well before birth in mammals (Pham, Gershon, & Rothman, 1991; M. Rao & Gershon, 2018). A recent study in mice showed that neurons in the myenteric plexus, though not the submucosal plexus, organize into “stripes” perpendicular to the longitudinal axis of the intestine – in the same orientation as circular muscle rings (Hamnett et al., 2021). Another study showed that specific planar cell polarity genes may be linked to enteric axon guidance, and that small changes in ENS organization can lead to large alterations in GI motility (Sasselli et al., 2013). However, relatively little is known about how the nascent neurons in both plexuses then establish the functional connections that enable them to sense the local environment as well as signal to other neurons and effector cells within the GI tract.

Neurons in the submucosal plexus, located directly beneath the mucosal layer of the intestinal wall, monitor luminal stimuli as well as regulate secretion and absorption; neurons in the myenteric plexus, located deeper within the two muscular layers of the intestinal wall, primarily control bowel motility patterns by coordinating intestinal contractions and relaxations (Avetisyan, Schill, & Heuckeroth, 2015; Foong, Tough, Cox, & Bornstein, 2014; Furness et al., 2014; Hansen, 2003). Within these two plexuses, there are approximately 20 distinct types of enteric neurons, which can be differentiated by function, morphology, connections, neurotransmitters, and electrophysiological signatures (Avetisyan et al., 2015; Furness, 2000). The myriad enteric neurons can be broadly divided into three main classes: intrinsic sensory neurons (commonly referred to as intrinsic primary afferent neurons [IPANs]), which detect and coordinate responses to

physical distension and chemical contents of the lumen; motor neurons, which can innervate the circular and longitudinal smooth muscle layers of the intestine to control intestinal motility, innervate the mucosa to induce secretions into the intestinal lumen, or innervate vessels to influence mucosal blood flow; and interneurons, which help to enable signaling between all classes of enteric neurons (Figure 1.1) (Avetisyan et al., 2015; Fung & Vanden Berghe, 2020; Furness, 2000; Furness et al., 2014; M. Rao & Gershon, 2016). Interneurons and motor neurons exhibit unipolar morphology and are also categorized as “S” or “Type I” neurons; while the sensory IPANs are multipolar and are often referred to as “AH” or “Type II” neurons (G. D. Hirst, Holman, & Spence, 1974).

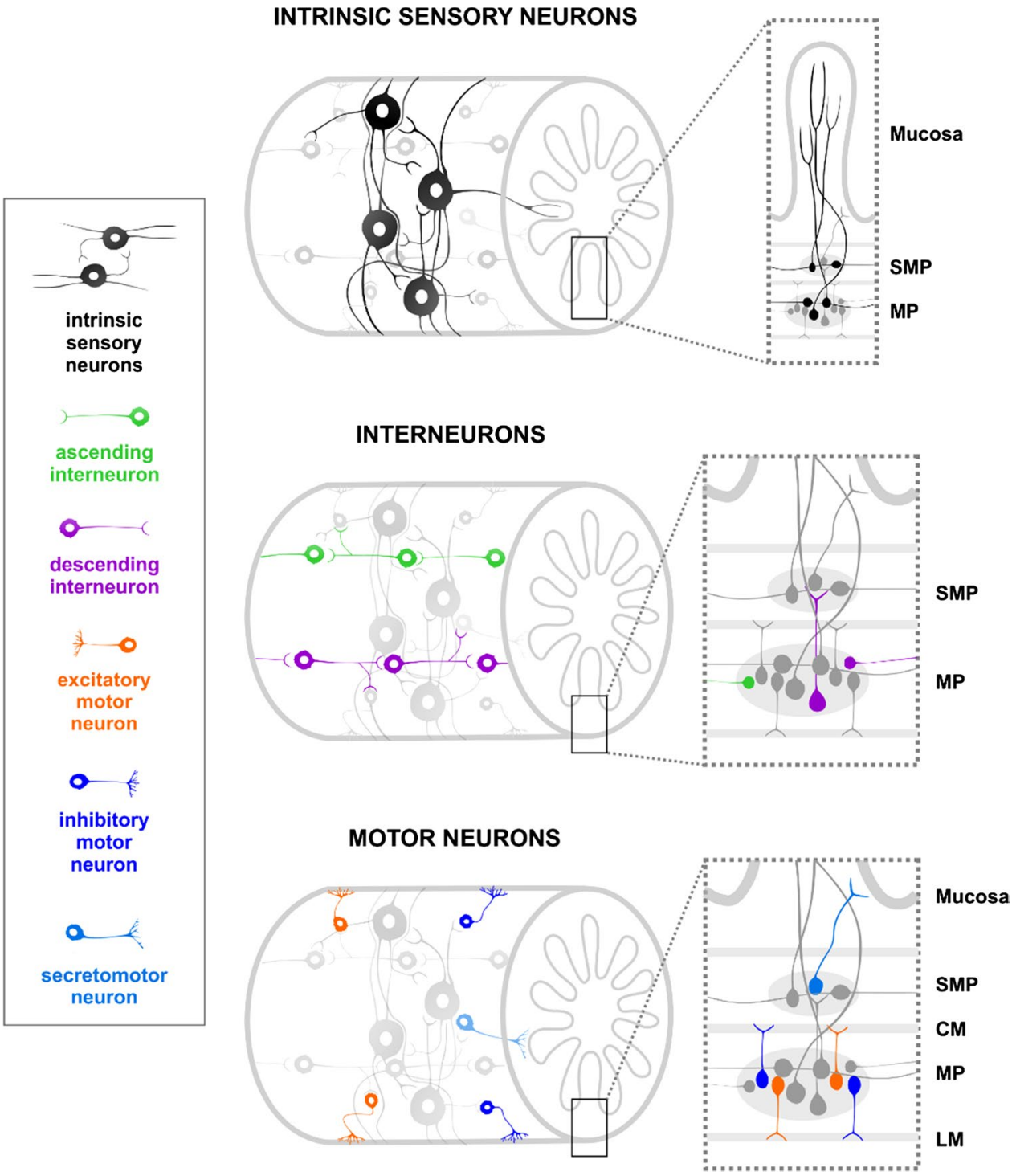


Figure 1.1: Overview of major enteric neuron subtypes. *Intrinsic sensory neurons (IPANS), with cell bodies in both the submucosal plexus (SMP) and myenteric plexus (MP) innervate the mucosa surrounding the intestinal lumen. In the MP, ascending interneurons, which innervate excitatory motor neurons, project orally while descending interneurons, which innervate inhibitory motor neurons, project anally. Excitatory and inhibitory motor neurons project to both the circular (CM) and longitudinal (LM) smooth*

muscle layers of the GI tract to produce coordinated contractions and relaxations. Caption modified from and figure reproduced from (Fung & Vanden Berghe, 2020), an open access article under the Creative Commons CC BY license.

Enteric neurons are not the only major excitable cell type localized in the GI tract. Chemo- and mechano-sensitive enterochromaffin cells, located in the intestinal epithelium, regulate fluid secretion (Alcaino et al., 2018; Linan-Rico et al., 2016; Strege et al., 2017). The muscular tissues of small and large bowel are made up of smooth muscle cells (SMCs) (Sanders, Koh, Ro, & Ward, 2012). SMCs are intrinsically excitable; however, in order to produce the stereotyped patterns of intestinal activity, SMCs must be modulated by enteric neurons as well as two types of interstitial cells: the interstitial cells of Cajal (ICCs), which are pacemakers for cyclical slow waves, and platelet-derived growth factor positive-α-receptor (PDGFRα^+) cells (Furness, 2000; Hansen, 2003; Huizinga et al., 1995; Sanders et al., 2012; Schneider, Wright, & Heuckeroth, 2019; Thomsen et al., 1998; Y. F. Zhu et al., 2014). These interstitial cells, which are also innervated by enteric neurons, electrically couple to circular and longitudinal SMCs via gap junctions to coordinate excitatory and inhibitory neural input with SMC contraction and relaxation (Cousins, Edwards, Hickey, Hill, & Hirst, 2003; Furness, 2000; Hansen, 2003; Horiguchi & Komuro, 2000; Huizinga et al., 1995; Sanders et al., 2012; Schneider et al., 2019).

Nearly all CNS neurotransmitters have also been found in ENS (Furness, 2000; Furness et al., 2014; M. Rao & Gershon, 2016). Secretomotor and vasomotor neurons control and coordinate intestinal secretions and blood flow, respectively. In response to mechanical and chemical luminal stimuli, secretomotor neurons primarily use vasoactive intestinal peptide (VIP) and substance P (SP) to signal to epithelial receptors and modulate

secretions and absorptions (Furness, 2007; Hansen, 2003; Hansen & Skadhauge, 1997; Reed & Vanner, 2001, 2003). Cholinergic vasomotor neurons ensure that mucosal blood flow is sufficient to fuel intestinal activities and allow for normal fluid exchange across the intestinal epithelium as water accompanies the influx of nutrient molecules from the lumen and efflux of electrolytes to the lumen (Furness, 2007; Hansen, 2003). Enterochromaffin cells synthesize 95% of the body's serotonin (5-HT), and they respond to mucosal stimulation by releasing 5-HT to IPANs (Alcaino et al., 2018; Bertrand, 2004; Linan-Rico et al., 2016; Strege et al., 2017). In turn, IPANs – which are immunoreactive for choline acetyltransferase (ChAT), an enzyme that synthesizes acetylcholine (ACh), as well as calbindin – stimulate interneurons (Furness, Costa, & Keast, 1984; Niel, 1991). Orally projecting, ascending interneurons stimulate excitatory motor neurons, while anally projecting, descending interneurons act on inhibitory motor neurons; both types of interneurons largely signal using ACh (Brookes, 2001; Furness, 2000; Furness & Costa, 1982; Li & Furness, 1998; Qu et al., 2008). Excitatory motor neurons are thought to release ACh, adenosine triphosphate (ATP), and tachykinins to interstitial cells and SMCs, while the major co-transmitters for the inhibitory motor neurons are nitric oxide (NO) and ATP (Furness, 2000; Greig & Cowles, 2017; Hansen, 2003; Qu et al., 2008; Schneider et al., 2019). ACh and tachykinins act on muscarinic ACh receptors (mAChRs) and neurokinin receptors on GI SMCs, respectively, to induce smooth muscle contraction (Cousins, Edwards, Hirst, & Wendt, 1993; Maggi et al., 1997). NO diffuses to local SMCs and induces smooth muscle relaxation through cyclic guanosine monophosphate (cGMP)-mediated signaling (Sanders & Ward, 1992). The neurotransmitter ATP is able produce either excitatory or inhibitory responses depending on the receptor present on

the postsynaptic cell; ligand-gated P2X receptors – along with nicotinic ACh receptors (nAChRs) – are localized to excitatory motor neurons, while G-protein-coupled P2Y receptors are found on inhibitory motor neurons (Galligan, LePard, Schneider, & Zhou, 2000; Giaroni et al., 2002; Ren & Bertrand, 2008).

Due to differences in neurotransmitter receptors and electrophysiological responses observed in ICCs versus PDGFRα^+ cells, it is thought that the two populations of interstitial cells are activated by different components of the enteric motor neuron signaling pathway. ICCs, which express mAChRs and neurokinin receptors, are thought to be the major recipients of cholinergic and signaling from excitatory motor neurons and the nitrergic signaling component from inhibitory motor neurons (Alberti et al., 2007; Iino & Horiguchi, 2006; Kito & Suzuki, 2003; Klein et al., 2013; Suzuki, Ward, Bayguinov, Edwards, & Hirst, 2003; X. Y. Wang, Paterson, & Huizinga, 2003). PDGFRα^+ cells can be differentiated from ICCs by their robust expression of purinergic signaling proteins, such as the $P2Y_1$ receptor, suggesting that these interstitial cells are the primary recipient of the ATP signaling component from inhibitory motor neurons (Kurahashi, Mutafova-Yambolieva, Koh, & Sanders, 2014; Peri, Sanders, & Mutafova-Yambolieva, 2013).

The excitatory and inhibitory motor neurons are the main drivers of intestinal contractions and relaxations, respectively. My thesis work aimed to understand the physiological causes of constipation and dysmotility in *SCN1B*-linked DEE as well as determine the role of *SCN1B* subunits in maintaining normal colonic motility; therefore, I will focus primarily on the activity of these excitatory and inhibitory motor neurons and how

temporally and spatially synchronized signaling between these two populations – both located in the myenteric plexus – produces the observed neurogenic motility pattens observed in the large intestine. My work will also touch on the IPANs and interstitial cells that mediate these motility responses.

Development and patterns of colonic motility

There are multiple distinct forms of motility that have been observed in the large intestine of humans and other mammals. These stereotyped, controlled smooth muscle movements are established by 1) “myogenic” activity that is intrinsic to GI smooth muscle and pacemaker cells and 2) “neurogenic” activity that is controlled by the inhibitory and excitatory motor neurons of the myenteric plexus and even influenced by external autonomic innervation (Spencer, Dinning, Brookes, & Costa, 2016).

It has been well-established that inhibitory signaling typically arises earlier in development than excitatory signaling in the mammalian ENS. Studies performed in neonatal guinea pigs suggest inhibitory neuromuscular transmission to longitudinal muscle in small intestine is stronger than in adult guinea pigs (Bian, Burda, Carrasquillo, & Galligan, 2009; Patel et al., 2010). While evidence of inhibitory ENS signaling to circular smooth muscle is already present at birth in mice, excitatory neuromuscular transmission to circular SMCs is not thought to develop until sometime between P6 and P10 – which coincides with the proportions of myenteric cholinergic neurons in murine small intestine finally reaching “adult” levels (Hao, Bornstein, & Young, 2013; Roberts, Murphy, Young, & Bornstein, 2007). Similarly, the proportion of nitrergic colonic myenteric neurons stabilizes

by P5 in rats, while the proportion of cholinergic enteric neurons continues to increase through P36 (de Vries, Soret, Suply, Heloury, & Neunlist, 2010). Though the precise development of enteric neuronal expression and activity is difficult to establish in humans, one study used dissected small intestine tissue from children (< 2 years old) and adults to compare *ex vivo* motility (Wittmeyer, Merrot, & Mazet, 2010). Though both adolescent and adult tissue exhibited spontaneous contractions, researchers found that NO neurotransmission exhibited a stronger, tonic inhibitory action in adolescent intestine (Wittmeyer et al., 2010). In summary, typical neurogenic patterns of intestinal motility are age-dependent, largely due to the delayed emergence of cholinergic enteric neurons and excitatory neuromuscular signaling.

Slow waves: Slow waves are spontaneous, rhythmic, undulating changes in the resting membrane potential of GI smooth muscle (M. K. K. Cheng H.M., Seluakumaran K. , 2020). These electrophysiological events are actively generated and propagated by the ICC network and then passively conducted to the surrounding SMCs via gap junctions (M. K. K. Cheng H.M., Seluakumaran K. , 2020; Furness, 2007; Sanders, 2019). Though slow waves in isolation are insufficient to induce smooth muscle contraction, input from the ENS and other external influences can result in the membrane potential reaching the “slow wave threshold” – at which point the smooth muscle membrane potential is sufficiently depolarized to initiate APs, cause an influx of calcium ions through open L-type voltage-gated calcium channels, and initiate smooth muscle contraction by excitation-contraction coupling (M. K. K. Cheng H.M., Seluakumaran K. , 2020; Sanders, 2019; Thorneloe & Nelson, 2005).

Colonic migrating motor complexes (CMMCs): CMMCs are neurally mediated; however, these cyclical motor patterns are thought to be less content-dependent and are therefore more prevalent in the fasted state (M. K. K. Cheng H.M., Seluakumaran K., 2020; Spencer et al., 2016). Unlike peristaltic contractions, CMMCs are capable of migrating both orally and arborally (Sanders, 2019; Sarna, Condon, & Cowles, 1984). The main purpose of this motility pattern is likely to clear the intestinal lumen of food remnants, residual secretions, and cellular debris in between meals (Beckett, Young, Bornstein, & Jadcherla, 2017). While the exact neurogenic signaling mechanisms underlying CMMCs are unclear, it has been proposed that these motility patterns are generated by either a reduction in tonic signaling from inhibitory motor neurons or are produced exclusively via synergistic signaling from excitatory motor neurons and ICCs (Brierley, Nichols, Grasby, & Waterman, 2001; Dickson, Heredia, McCann, Hennig, & Smith, 2010; Roberts et al., 2007; Spencer, 2001).

Peristalsis: The peristaltic reflex is a neurally mediated contractile response to luminal content (Figure 1.2). Fecal material or a “bolus” in the large intestine can mechanically and chemically activate enterochromaffin cells in the lumen epithelium, causing these cells to release 5-HT to IPANS (Alcaino et al., 2018; Bertrand, 2004; Linan-Rico et al., 2016; Strege et al., 2017). There is also evidence that certain excitatory and inhibitory interneuron populations are mechanosensitive to specific types of stretch and that their signaling complements that of the IPANs during the peristatic reflex (Smith, Spencer, Hennig, & Dickson, 2007). This signaling pathway(s) ultimately results in both excitatory

and inhibitory neuronal signaling to SMCs in both circular and longitudinal GI muscle layers, which moves the material in an aboral direction via a polarized reflex response (Spencer et al., 2016). In the segment directly distal to the bolus, smooth muscle relaxation produces an expanded GI "pocket" that receives the moving materials (Spencer, Hennig, & Smith, 2003; Wood, 2004). In the intestinal segment directly proximal to the luminal content, smooth muscle contractions propel the contents to the newly formed pocket (Spencer et al., 2003; Wood, 2004). Once the bolus is pushed to its new, more distal location in the intestinal lumen, it activates the same reflex response again; this pattern continues until the material exits the intestinal tract (Spencer et al., 2016).

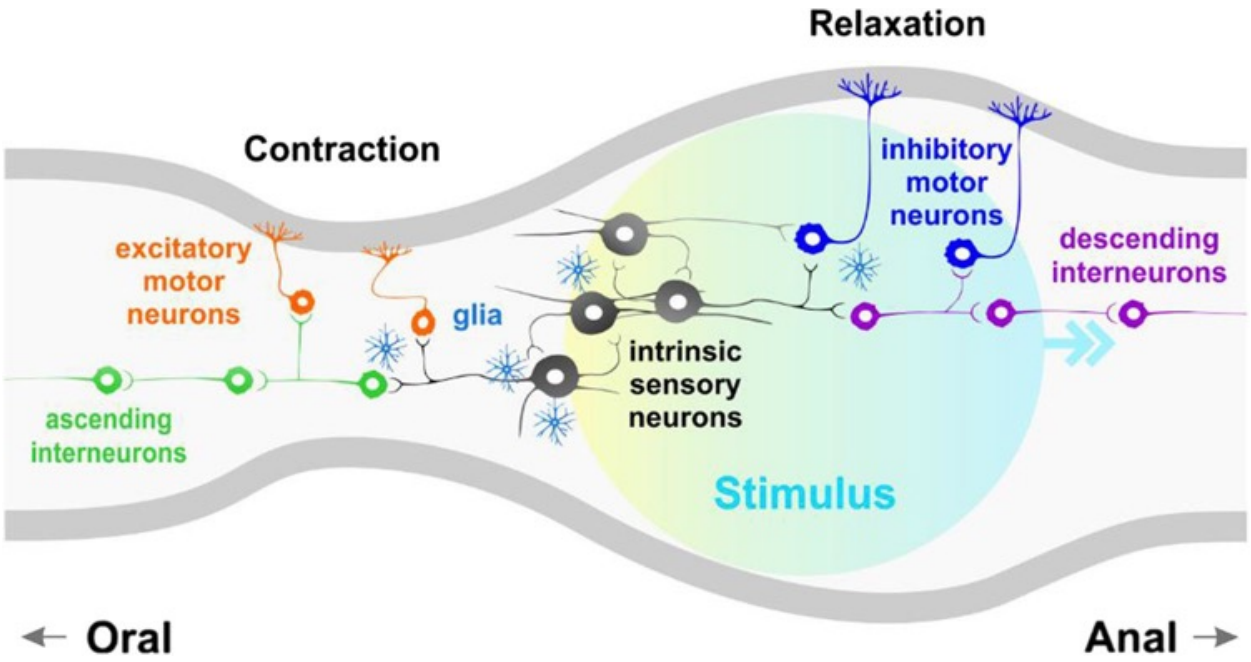


Figure 1.2: An overview of the peristaltic reflex. *Intrinsic sensory afferents, detecting a stimulus in the intestinal lumen, signal to ascending and descending interneurons. Excited by the ascending interneurons, excitatory motor neurons signal to circular and longitudinal muscle to produce an oral contraction; in tandem, inhibitory motor neurons – innervated by descending interneurons, produce an anal relaxation. Caption modified from and figure reproduced from (Fung & Vanden Berghe, 2020), an open access article under the Creative Commons CC BY license.*

The patterns of colonic motility – and thus the proper functioning of the enteric neurons, interstitial pacemaker cells, and effector SMCs that initiate and control them – are vital for normal digestion and excretion. In turn, the excitability and signaling capacity of these ENS cell populations is largely dependent on VGICs.

Overview of voltage-gated ion channels in ENS development and intestinal motility

VGSCs: Electrophysiological recordings from human intestinal pacemaker cells (Strege, Ou, et al., 2003) and smooth muscle (Holm et al., 2002) have shown that TTX-R VGSC activity is likely involved in regulating intestinal electrical slow waves and subsequently smooth muscle contractions. Patient variants in *SCN5A* have been shown to alter I_{Na} density as well as voltage-dependence and VGSC mechanosensitivity in experiments performed in heterologous cells (Saito et al., 2009; Strege et al., 2018). Furthermore, ranolazine, a drug that selectively blocks $Na_v1.5$-mediated I_{Na} and causes constipation in patients, was shown to reduce peak I_{Na} and VGSC mechanosensitivity in isolated human colonic smooth muscle cells as well as decrease both basal and stimulation-induced contractility in human colonic muscle segments (Neshatian et al., 2015). An analysis of colon tissue samples taken from patients with Hirschsprung's disease, a condition characterized by problems passing stool, revealed decreased $Na_v1.9$ protein expression relative to control human colon samples (O'Donnell et al., 2016).

Work in animal models suggests that $Na_v1.5$ and $Na_v1.9$ play important roles in controlling the firing patterns of mammalian enteric neurons, with both VGSCs being expressed by

enteric motor neurons and $Na_v1.9$ being localized primarily to IPANS (Osorio, Korogod, & Delmas, 2014). More specifically, a study performed in mice lacking $Na_v1.9$ showed altered colonic contractility patterns, suggesting that intestinal VGSC activity is crucial for regulating gut motility in mammals (Copel, Clerc, Osorio, Delmas, & Mazet, 2013). There is also substantial evidence for TTX-S VGSC α subunit involvement in in both Type I and Type II enteric neuron action potential generation as well as patterns of colonic motility, due to the striking observed effect of TTX on these activities (Fida, Lyster, Bywater, & Taylor, 1997; G. D. Hirst et al., 1974; Nakagawa, Misawa, Nakajima, & Takaki, 2005). The exact identities of the TTX-S VGSC α subunits that contribute to enteric neuron action potentials have not been verified, but mRNA expression of *Scn2a*, *Scn3a*, *Scn8a*, and *Scn9a* has been observed in mammalian small and large intestine (Bartoo, Sprunger, & Schneider, 2005; Sage et al., 2007). Additionally, epithelial enterochromaffin cells are also known to express $Na_v1.3$, which is thought to be important for their excitability and 5-HT release (Strege et al., 2017).

Voltage-gated calcium channels (VGCCs): In addition to the L-type Ca_v channels (Ca_v1) which enable smooth muscle contraction, VGCCs are also localized to interstitial cells and enteric neurons. T-type VGCCs (Ca_v3) are expressed by murine ICCs, where they are important for synchronizing pacemaker activity and propagating slow waves (Beyder & Farrugia, 2012; Y. C. Kim, Koh, & Sanders, 2002; Zheng, Park, Koh, & Sanders, 2014). $Ca_v2.2$ (N-type) is the predominant VGCC expressed by mammalian enteric neurons and are thought to largely control ACh release in myenteric plexus; $Ca_v2.3$ (R-type) also plays a role in ENS signaling – more specifically, in controlling release of particular

neurotransmitters from inhibitory motor neurons (Bian, Zhou, & Galligan, 2004; Rugiero et al., 2002; Shuttleworth & Smith, 1999; Smith, Kang, & Vanden Berghe, 2003; Vogalis, Harvey, Lohman, & Furness, 2002; Wessler, Dooley, Werhand, & Schlemmer, 1990). It is thought that inhibitory motor neurons co-release ATP and NO to induce smooth muscle relaxations (Mane et al., 2014). $Ca_v2.2$ is postulated to mediate the release of ATP from inhibitory motor neurons, while $Ca_v2.3$ drives the release of NO (Bridgewater, Cunnane, & Brading, 1995; E. S. Rodriguez-Tapia, Naidoo, DeVries, Perez-Medina, & Galligan, 2017).

Voltage-gated potassium channels (VGKCs): The enteric nervous system (ENS) develops from enteric neural crest-derived cells (ENCCs) that migrate to the foregut during mammalian embryonic development (Hao et al., 2012; C. S. Hirst et al., 2015). Researchers have noted robust $K_v3.4$ expression on neurites close to the murine ENCC migratory wavefront, but there is no solid evidence that these channels influence ENCC migration or generation of enteric neurites (C. S. Hirst et al., 2015). Kv1 and Kv4 channel activity and/or expression have been found in ICCs and GI SMCs (Amberg, Koh, Imaizumi, Ohya, & Sanders, 2003; Beyder & Farrugia, 2016; Hart et al., 1993; Hatton et al., 2001; Y. C. Kim et al., 2002; Overturf et al., 1994; Parsons & Huizinga, 2010). Additionally, the Kv7 channel family is thought to play a role in mediating noxious chemical and mechanical stimuli in the GI tract (Peiris et al., 2017).

1.4 Conclusions

The ENS is a vast, complex physiological system that relies on a plethora of cell types, local connections, and signaling pathways to develop and operate properly. Many of these foundational mechanisms are similar to those that ensure appropriate functioning of the CNS, and there are many examples of comorbid CNS and ENS dysfunction in the patient population. VGSC α and β1 subunits influence cell excitability, neuronal development and patterning, and the expression patterns of other ion channel genes. Variants in *SCN1B* alter neuronal activity and cause various forms of developmental delay; recently, *SCN1B* was also linked to ENS function in the patient population. There are many anecdotal reports and clinical case studies suggesting that VGSC-associated gene variants – including *SCN1B* – are linked to GI dysfunction, including dysmotility and constipation. However, the precise role of VGSC β1 subunits in GI motility remains unknown. The research presented in Chapter 2 of this thesis provides a strong rationale for the direct involvement of ENS-localized VGICs in the colonic dysmotility experienced by many DEE patients. Subsequently, in Chapter 3 I assess the specific effects of *Scn1b* deletion in the development, organization, and function of the colonic myenteric plexus in a mouse model of DEE.

Chapter 2 : Gastrointestinal Symptomology in Developmental and Epileptic Encephalopathy Patients

A modified version of this chapter has been published:

***Beck V.C.**, Isom L.L., Berg A.T. Gastrointestinal symptoms and channelopathy-associated epilepsy. J Pediatr. 2021 Jun 25:S0022-3476(21)00616-8. doi:10.1016/j.jpeds.2021.06.034. Epub ahead of print. PMID: 34181986.*

2.1 Abstract

The objective of this work was to determine the prevalence of and identify factors associated with gastrointestinal (GI) symptoms among patients with channelopathy-associated developmental and epileptic encephalopathy (DEE). Parents of 168 patients with DEEs linked to *SCN1A* (N=59), *KCNB1* (N=31), or *KCNQ2* (N=78) completed online CLIRINX© surveys about their children's GI symptoms. Analysis examined prevalence, frequency, and severity of GI symptoms, as well as DEE type, functional mobility, feeding difficulties, ketogenic diet, anti-seizure medication, autism spectrum disorder (ASD), and seizures. Statistical analyses included chi-square tests, Wilcoxon rank-sum analyses, and multiple logistic regression.

GI symptoms were reported in 92/168 (55%) patients among whom 63/86 (73%) reported daily or weekly symptoms, 29/92 (32%) had frequent or serious discomfort, and 13/91 (14%) had frequent or serious appetite disturbances as a result. The prevalence of GI

symptoms varied across DEE cohorts with 44% of *SCN1A*-DEE patients, 35% of *KCNB1*-DEE patients, and 71% of KCNQ2-DEE patients reporting GI symptoms in the previous month. After adjustment for DEE type, current use of ketogenic diet (6% reported), and gastrostomy tube (13% reported) were both associated with GI symptoms in a statistically, but not clinically significant manner ($P < 0.05$). Patient age, functional mobility, feeding difficulties, ASD, and seizures were not clearly associated with GI symptoms. Overall, no individual anti-seizure medication was significantly associated with GI symptoms across all DEE cohorts.

GI symptoms are common and frequently severe in DEE patients. Understanding GI symptoms in DEEs is essential for guiding future work to investigate how variants in voltage-gated ion channel genes may directly contribute to GI pathology via alterations in GI cell function.

2.2 Introduction

Patients with developmental and epileptic encephalopathies (DEEs) often exhibit a wide range of impairments, which can complicate treatment and reduce quality of life (A. T. Berg, Tarquinio, & Koh, 2017). One often distressing concern that is repeatedly found in clinical reports of patients with DEE, including those with variants in genes encoding voltage-gated ion channels, is gastrointestinal (GI) dysfunction, particularly constipation and dysmotility (Berecki et al., 2018; A. T. Berg et al., 2017; Cannon et al., 2016; Dabby et al., 2016; Fertleman et al., 2006; Gardella & Møller, 2019; Gay et al., 2008; Hisama et al., 1993; Howell et al., 2015; Leipold et al., 2013; Srivastava et al., 2018; Villas et al., 2017; Wirrell et al., 2017). These anecdotal reports, however, have not been systematically explored or characterized.

Multiple factors may be linked to the development of GI symptoms in the DEE patient population. Physical inactivity contributes to constipation in the general population (Center, 2018; S. S. Rao & Go, 2010), and many patients with DEE often have limited mobility (A.T. Berg et al., 2020). The ketogenic diet, used as a treatment for intractable seizures in many DEE patients, as well as the use of enteral nutrition in DEE patients with feeding difficulties, are potential causes of GI symptoms (Bittencourt et al., 2012; Nei, Ngo, Sirven, & Sperling, 2014; Vezyroglou & Cross, 2016). Many anti-seizure medications are associated with adverse GI side-effects (Jahromi et al., 2011). Finally, many DEE patients have also been diagnosed with autism spectrum disorder (ASD) (Srivastava & Sahin, 2017), which is frequently accompanied with increased reports of GI symptoms,

with constipation being most common (McElhanon, McCracken, Karpen, & Sharp, 2014; Ofei & Fuchs, 2018). The etiology of the GI disturbances observed in ASD patients is not known; although, as with DEE patients, decreased physical mobility, diet, and side-effects of medications have been postulated as potential causes (McElhanon et al., 2014; Ofei & Fuchs, 2018). Conversely, there is anecdotal clinical evidence as well as animal studies suggesting that constipation can induce seizure activity (Moezi, Pirsalami, & Inaloo, 2015).

Ion channel gene-linked DEE variants may play direct roles in GI dysfunction. Voltage-gated sodium (Na_v) and potassium (K_v) channels are expressed in the mammalian GI tract, where they regulate the activity of enteric neurons and other excitable cells, thereby contributing to proper GI motility and fecal expulsion (Copel et al., 2013; Eijkenboom et al., 2019; Hatton et al., 2001; Hetz et al., 2014; Holm et al., 2002; Jepps et al., 2009; McKay, Ye, & Huizinga, 2006; Neshatian et al., 2015; O'Donnell et al., 2016; Osorio et al., 2014; Ou et al., 2002; Saito et al., 2009; Strege, Holm, et al., 2003; Strege, Ou, et al., 2003). Thus, DEE-linked genetic variants may potentially directly affect GI function by causing aberrant GI cell excitability.

There is an unmet clinical need to understand the mechanism(s) of GI symptoms in DEE. As a first step toward this goal, we used responses from a parent survey that included a large number of children with DEEs linked to variants in *SCN1A*, *KCNB1*, or *KCNQ2* (which encode for the voltage-gated ion channels, $Na_v1.1$, $K_v2.1$, and $K_v7.2$, respectively) to uncover potential associations between the presentation of GI symptoms and known

risk factors for GI dysfunction: mobility, diet, medication, and ASD. An improved understanding of the factors and biological mechanism(s) underlying the recurring GI symptoms observed in DEE patients may lead to the development of novel treatments that address this disruptive comorbidity and improve quality of life for patients and caregivers.

2.3 Methods

Survey design and participation

An online parent-reported survey was performed using the CLIRINX® platform (Dublin, Ireland) and disseminated to families through several organizations including the Dravet Syndrome Foundation, the *KCNB1* family group, and the *KCNQ2* Cure Alliance. Caregivers of DEE patients of all ages were invited to participate. All survey data were collected through parent reports from June 2018 through February 2020. Parents were invited to upload their children's genetic testing reports; however, we based inclusion of a child in this study on the parent's affirmation that the child had a causative variant in one of the three ion channel genes of interest: *SCN1A, KCNB1,* or *KCNQ2*. Full methods for the survey have been previously described (A.T. Berg et al., 2020). The following provides a synopsis.

Gastrointestinal (GI) symptoms

Parents were asked to indicate whether their child had experienced specific GI symptoms—constipation, gut dysmotility or diarrhea—over the past month. They could also write in additional symptoms. As virtually all of the symptoms endorsed or reported were either constipation or dysmotility, and these two symptoms overlapped in all but a few cases, we simplified our primary outcome to the reporting of "constipation, dysmotility, or both" versus "neither". For children with GI symptoms in the previous month, parents reported typical frequency ("daily", "weekly", "monthly", or "less than once per month") as well as whether the symptoms caused either discomfort or affected the child's appetite

("no", "yes – occasionally but not a serious concern", or "yes – common occurrence or serious concern"). Parents also reported if they had used any specific methods to alleviate the GI symptoms (supplements, enemas, medications).

Potential risk factors associated with GI symptoms

Parents provided information concerning functional mobility (for children $\geq$ 2 years), dietary therapies, feeding tube usage, current anti-seizure medications, and diagnosis or features of autism.

For mobility, we used the Gross Motor Functional Classification System (GMFCS) (Morris, Galuppi, & Rosenbaum, 2004). For analytic purposes, the GMFCS scale was condensed into three levels: "dependent" (scores of 4 or 5), walks but "requires aids" (score of 3), and "independent" (scores of 1 or 2).

Parents were asked if their child had a feeding tube and, if so, whether their child was exclusively tube-fed or partially tube-fed. For children who ate by mouth (including those partially tube fed), parents indicated how often a child had difficulty chewing or swallowing. Choices were: "multiple times per meal", "usually once per meal", "not every meal but at least once on most days", "not daily but usually a few times per week", "less than once per week but every now and then", or "almost never". These responses were condensed into the categories: "typically at least once per meal", "typically more than once per day", "typically more than once per week", or "rarely or never".

Parents were also asked whether their child was currently being treated with a classic ketogenic, modified Atkins, or other special diet.

Parents reported if their child had an ASD diagnosis or presented with features of ASD. Parents reported on the occurrence of seizures in the previous six months and whether bowel movements were considered by the parents to be a trigger for seizures.

Finally, parents reported the child's current use of 35 specific anti-seizure medications. For overall analysis of all three DEE cohorts, only medications being taken by 10 or more patients were considered. For individual analysis of each DEE cohort, only medications that were being taken by five or more patients were considered.

Data analysis

Statistical analyses were performed in SAS©. Methods included Chi-square tests, Wilcoxon rank-sum analyses, and multiple logistic regression. Relative risks were used to quantify the strength of associations where appropriate. Results were considered significant at $P < 0.05$.

IRB approval and informed consent

All procedures for this study were approved by the Lurie Children's Hospital IRB. Parents provided informed consent electronically through the CLIRINX® platform.

2.4 Results

Participants

Of parents who enrolled in the study, 168 affirmed their child's pathogenic variant in one of the three ion channel genes of interest, *SCN1A* (N=59), *KCNB1* (N=31), or *KCNQ2* (N=78), and completed the survey portions about GI symptoms. Median patient age at the time of survey participation was 6.3 years (Interquartile Range: 3.3 to 10.3 years), and 55% of affected children were female (Table 2.1). The overall age distribution of DEE patients was: <5 (43%), 5-9 (30%), 10-15 (15%), ≥ 16 years (12%). The children in *KCNQ2*-DEE group had a substantially younger age than those in the other two groups ($P < 0.001$). The sex distribution was similar across groups ($P = 0.97$; Table 2.1).

Table 2.1: Demographic characteristics of DEE Cohorts

	N	***SEX N (% FEMALE)***	***MEDIAN AGE IN YEARS (IQR / RANGE)***
Total	168	92 (55%)	6.3 (3.3 to 10.3 / 0.2 to 37.2)
SCN1A-DEE (Dravet Syndrome)	59	33 (56%)	8.1 (4.8 to 16.3 / 0.4 to 37.2)
KCNB1-DEE	31	17 (55%)	8.2 (4.5 to 12.0 / 2.6 to 22.9)
KCNQ2-DEE	78	42 (54%)	4.5 (2.0 to 8.4 / 0.2 to 20.3)

Most participants (127/168, 76%) were from North America, 27/168 (16%) were from Europe or the United Kingdom, and 14/168 (8%) were from other countries or did not specify. The majority of patients with DEE self-identified White or Caucasian (154/168; 92%). Other reported races were Asian (9/168; 5%); American Indian, Alaskan Native,

First Nations (1/168; 0.005%); Black or African American (1/168; 0.005%), and Native Hawaiian or other Pacific Islander (1/168; 0.005%). Twelve (7%) parents reported that their child was of Hispanic or Latin ethnicity.

Gastrointestinal symptoms

Overall, 92/168 (55%) parents reported constipation or dysmotility in their child. The prevalence of GI symptoms differed significantly across the three epilepsy groups (Table 2.2). Despite the similar distributions of males and females between the three cohorts (Figure 2.1A), GI symptoms were not clearly associated with sex ($P = 0.08$). We explored sex differences within each DEE group and found, within the *SCN1A*-DEE cohort, a substantial association between GI symptoms and sex [20/33 (61%) female *SCN1A*-DEE patients versus 6/26 (23%) male patients, ($P = 0.004$; Figure 2.1B)]. No such association was apparent in the other two DEE groups. Despite age differences across the DEE groups, the age distributions of patients with and without GI symptoms were very similar with median ages of 6.2 and 6.3 years, respectively (Figure 2.1C). Furthermore, after adjustment for DEE type, patient age was not significantly associated with the manifestation of GI symptoms ($P = 0.09$, Figure 2.1D).

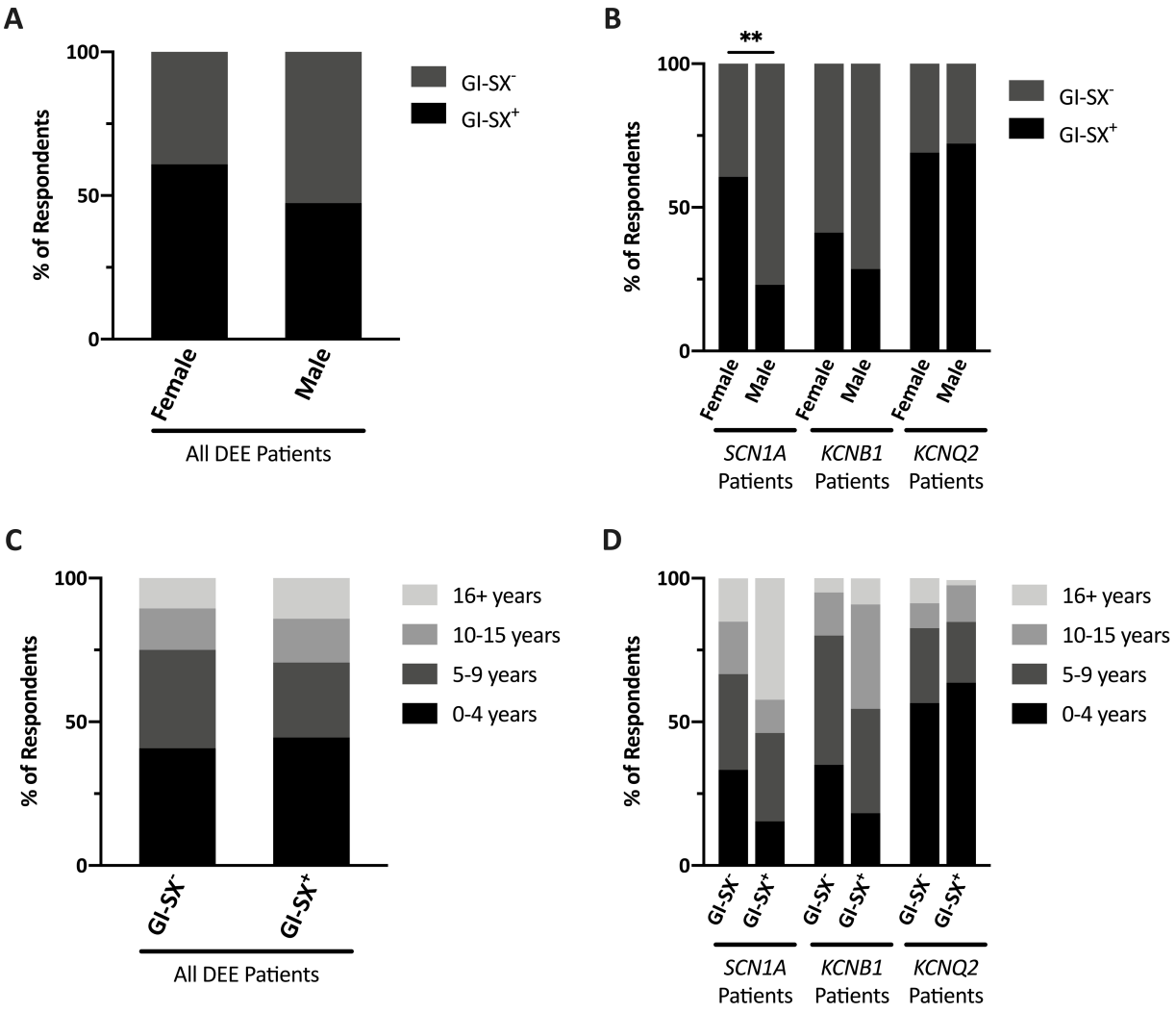


Figure 2.1: Sex and age distributions of patients with reported GI symptoms (GI-SX+) and those with no reported GI symptoms (GI-SX-) within SCN1A-, KCNB1-, or KCNQ2-DEE cohorts. *(A) The distribution of females and males did not significantly differ overall between the GI-SX+ and GI-SX- patient groups. (B) There were proportionally more females than males with GI symptoms within the SCN1A cohort, but there were no significant differences between the proportions of males and females with GI symptoms in the KCNB1 or KCNQ2 cohorts. (C, D) The age distribution did not significantly differ between GI-SX+ and GI-SX- patient groups in any of the three cohorts. ** = P < 0.01.*

Most parents (86/92) who reported GI symptoms in their children also provided information on how frequently these symptoms manifested. Survey responders reported either daily or weekly symptoms in 63/86 (73%) DEE patients, overall. Daily or weekly symptoms were reported in 15/25 (60%) *SCN1A*-DEE patients*,* 8/11 *(*73%) *KCNB1*-DEE patients, and in 40/50 (80%) *KCNQ2*-DEE patients (Table II).

Patient discomfort, stemming from the GI symptoms, was considered a common occurrence or serious concern in 29/92 (32%) affected patients (Table II). In a post-hoc comparison, *SCN1A*-DEE patients were significantly less likely than *KCNB1*- and *KCNQ2*-DEE patients (combined) to experience common or serious discomfort as a result of their GI symptoms ($P = 0.02$). Parent-reported changes to appetite due to GI symptoms was a common occurrence or serious concern for 13/91 (14%) of responders (Table 2.2). *KCNB1*-DEE patients were marginally more likely to experience appetite disturbances as a consequence of GI symptoms, compared to the other two groups ($P = 0.06$).

Multiple treatments were utilized by parents to relieve the GI symptoms; 40/92 (43%) reported using including dietary supplements, 19/92 (21%) reported using enemas, 44/92 (48%) used medications, and 32/92 (35%) reported using other therapeutics.

Table 2.2: Distribution of GI-related measures within DEE cohorts

	***SCN1A*-DEE (Dravet Syndrome)**	***KCNB1*-DEE**	***KCNQ2*-DEE**	**Across-group differences**
GI symptom prevalence	(26/59) 44%	(11/31) 35%	(55/78) 71%	*P* < 0.001
GI symptom frequency				*P* = 0.14
% daily	(4/25) 16%	(2/11) 18%	(18/50) 36%	
% weekly	(11/25) 44%	(6/11) 55%	(22/50) 44%	
% monthly	(5/25) 20%	(2/11) 18%	(9/50) 18%	
% <1 day/month	(5/25) 20%	(1/11) 9%	(1/50) 2%	
GI symptom effects				
% discomfort	(20/26) 77%	(10/11) 91%	(50/55) 91%	*P* = 0.09
[a] % Yes, common occurrence/serious concern	(3/26) 12%	(4/11) 36%	(22/55) 40%	
[b] % Yes, occasionally but not a serious concern	(17/26) 65%	(6/11) 55%	(28/55) 51%	
% appetite disturbances	12/26) 46%	(8/10) 80%	(32/55) 58%	*P* = 0.10
[a] % Yes, common occurrence/serious concern	(2/26) 8%	(4/10) 40%	(7/55) 13%	
[b] % Yes, occasionally but not a serious concern	(10/26) 38%	(4/10) 40%	(25/55) 45%	
Functional mobility				*P* < 0.001
% dependent	(2/54) 4%	(6/31) 19%	(21/50) 42%	
% requires aids	(2/54) 4%	(4/31) 13%	(7/50) 14%	
% independent	(50/54) 92%	(21/31) 68%	(22/50) 44%	
Ketogenic diet (KD)				*P* = 0.13
% currently using KD	(3/59) 5%	(4/31) 13%	(2/70) 3%	
% not currently using KD	(56/59) 95%	(27/31) 87%	(68/70) 97%	
Gastrostomy tube				*P* = 0.28
% exclusive	(2/59) 3%	(4/31) 13%	(6/70) 9%	
% partial use	(4/59) 7%	(3/31) 10%	(2/70) 3%	
% never	(53/59) 90%	(24/31) 77%	(62/70) 88%	
Chewing/swallowing difficulties				*P* = .0018
% every meal	(8/57) 14%	(0/26) 0%	(14/64) 22%	
% daily	(2/57) 4%	(5/26) 19%	(17/64) 26%	
% weekly	(7/57) 12%	(4/26) 15%	(5/64) 8%	
% <1 day/week	(40/57) 70%	(17/26) 66%	(28/64) 44%	
Autism diagnosis/features	(30/59) 51%	(15/31) 48%	(27/70) 39%	*P* = 0.35
Seizure prevalence	(56/59) 95%	(23/31) 74%	(26/59) 44%	*P* < 0.001

Mobility limitations and GI symptoms

The distribution of functional mobility scores differed significantly across the three cohorts (Table 2.2). However, using multiple logistic regression to control for the effect of individual DEEs, we found no overall association between functional mobility and presence of GI symptoms (P = 0.47, Figure 2.2A). Notably, although *KCNQ2*-DEE patients exhibited both the lowest functional mobility scores (P < 0.001) as well as the highest prevalence of GI symptoms among the three cohorts, within the *KCNQ2*-DEE group, there was no significant association between mobility scores and GI symptoms (P = 0.23).

Ketogenic diet and GI symptoms

In total, 9/160 (6%) children were reported to be currently treated with the ketogenic diet (Table 2.2. On bivariate analysis, use of the ketogenic diet was not associated with GI symptoms (P = 0.14); however, after adjustment for DEE, use of ketogenic diet was significantly associated with GI symptoms (P = 0.03). This change appears to be due to a finding in the *KCNB1* cohort; all children in that group who were using the ketogenic diet also had GI symptoms (Figure 2B).

Gastrostomy tube and GI symptoms

Current partial or exclusive use of a gastrostomy tube was reported in 21/160 (13%) DEE patients and did not differ significantly between DEE groups (Table II). After adjusting for DEE, use of gastrostomy tube was found to be significantly associated with GI symptoms (P = 0.03; Figure 2C).

Chewing and swallowing difficulties and GI symptoms

Excluding patients who were exclusively tube-fed, frequent (occurring at least once per meal) difficulties with chewing or swallowing were reported for 22/147 (15%) DEE patients (Table II). The frequency of reported chewing or swallowing difficulties varied significantly across the three DEE groups (Table II), with *KCNB1*-DEE patients significantly less likely to have chewing or swallowing difficulties ($P = 0.003$). After adjusting for DEE, chewing and swallowing difficulties were not associated with reports of GI symptoms ($P = 0.52$; Figure 2D).

Autism spectrum disorder (ASD) and GI symptoms

Either a diagnosis or having features of ASD was reported in 72/160 (45%) DEE patients; this did not vary across the three DEE groups (Table II). After adjusting for DEE, there was no significant association between reported ASD diagnosis or features and GI symptoms ($P = 0.08$; Figure 2.2E).

Seizures and GI symptoms

While seizure prevalence in the previous six months differed significantly between the different DEE groups (Table 2.2), there was no correlation between seizures and GI symptoms after adjusting for DEE type ($P = 0.99$; Figure 2.2F). Overall, 15/135 (11%) patients were reported to have bowel movements as a seizure trigger, and DEE patients with GI symptoms were significantly more likely to report seizures triggered by bowel movements ($P = 0.02$).

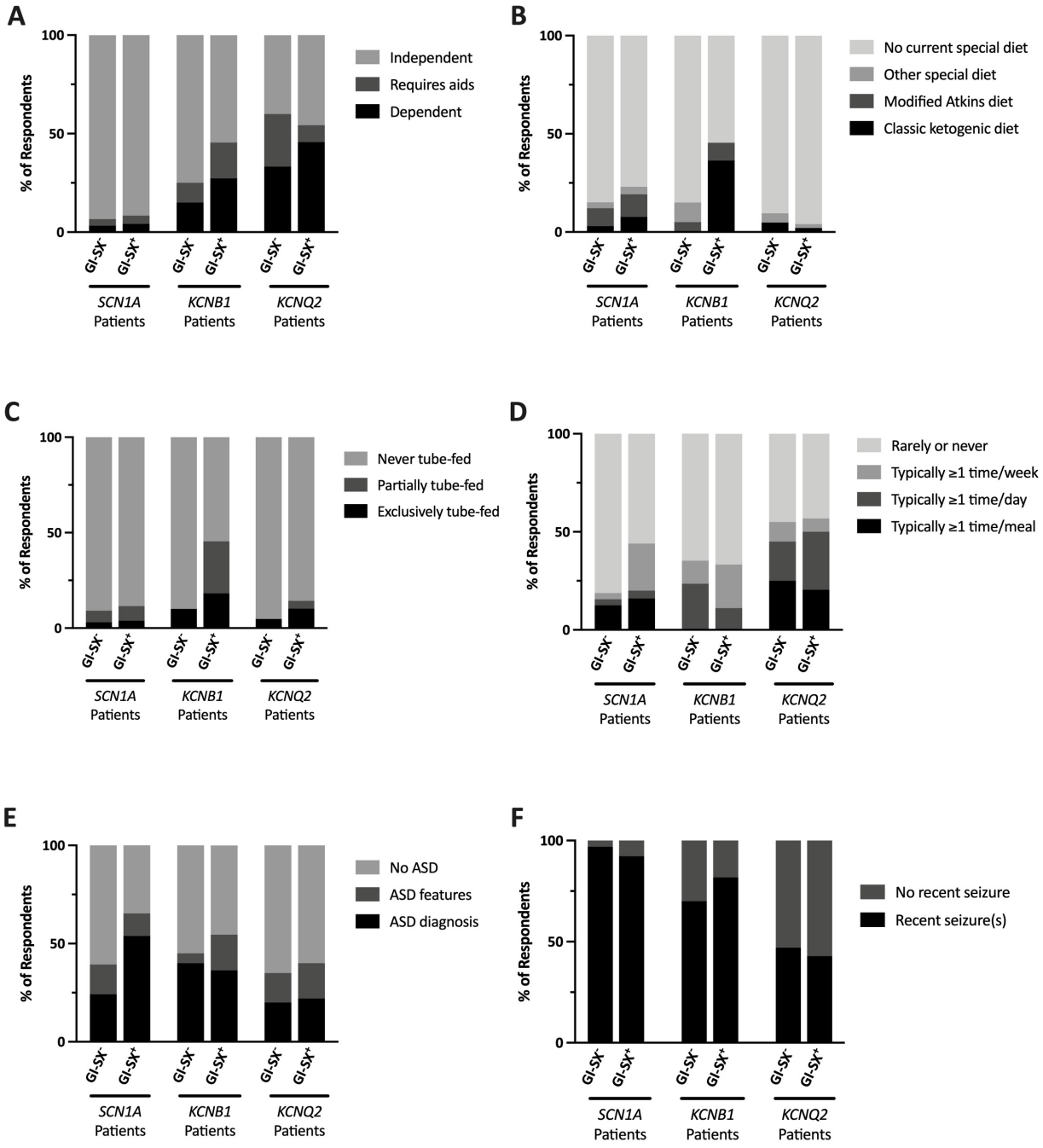
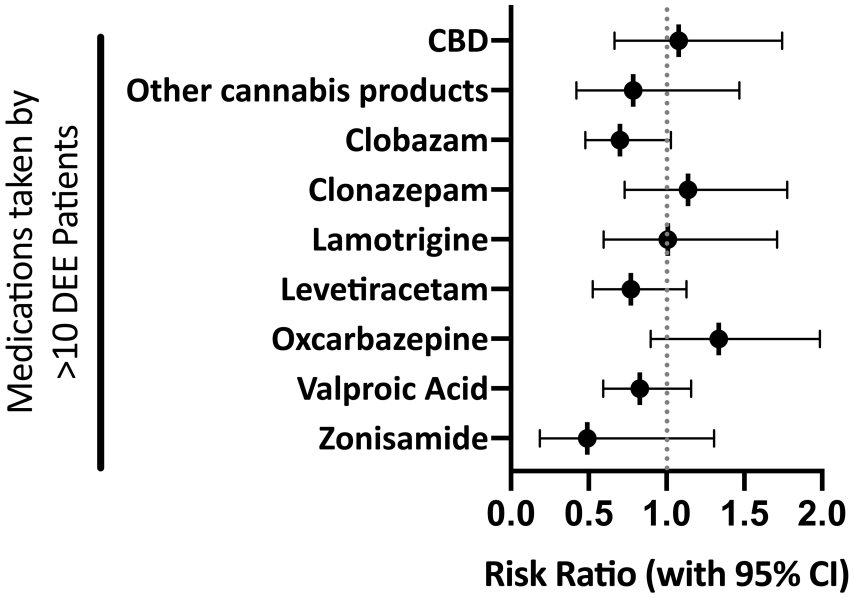


Figure 2.2: Distributions of SCN1A-, KCNB1-, or KCNQ2-DEE patient functional domains in patient groups with (GI-SX+) and without (GI-SX-) GI symptoms*. Parents reported information regarding the (A) degree of independent mobility, (B) current use of a special diet, (C) relative reliance on a gastrostomy tube for feeding, (D) frequency of chewing and/or swallowing difficulties, (E) autism spectrum disorder (ASD) diagnosis or features in their child, and (F) occurrence of any seizures in the previous six months.*

Anti-seizure medications and GI symptoms

Overall, no individual medication appeared to be linked with GI symptoms across the three DEE groups (Figure 2.3A). In exploratory analyses within each cohort (Figure 2.3B); we found stiripentol, a drug specifically approved for Dravet syndrome, was significantly associated with increased report of GI symptoms in *SCN1A*-DEE patients ($P < 0.001$). We also found a significant association between cannabidiol (CBD) and GI symptoms in *KCNB1*-DEE patients ($P = 0.006$) but not the other groups.

A


Medications taken by
>10 DEE Patients
CBD
Other cannabis products
Clobazam
Clonazepam
Lamotrigine
Levetiracetam
Oxcarbazepine
Valproic Acid
Zonisamide
0.0
0.5
1.0
1.5
2.0
Risk Ratio (with 95% CI)

B

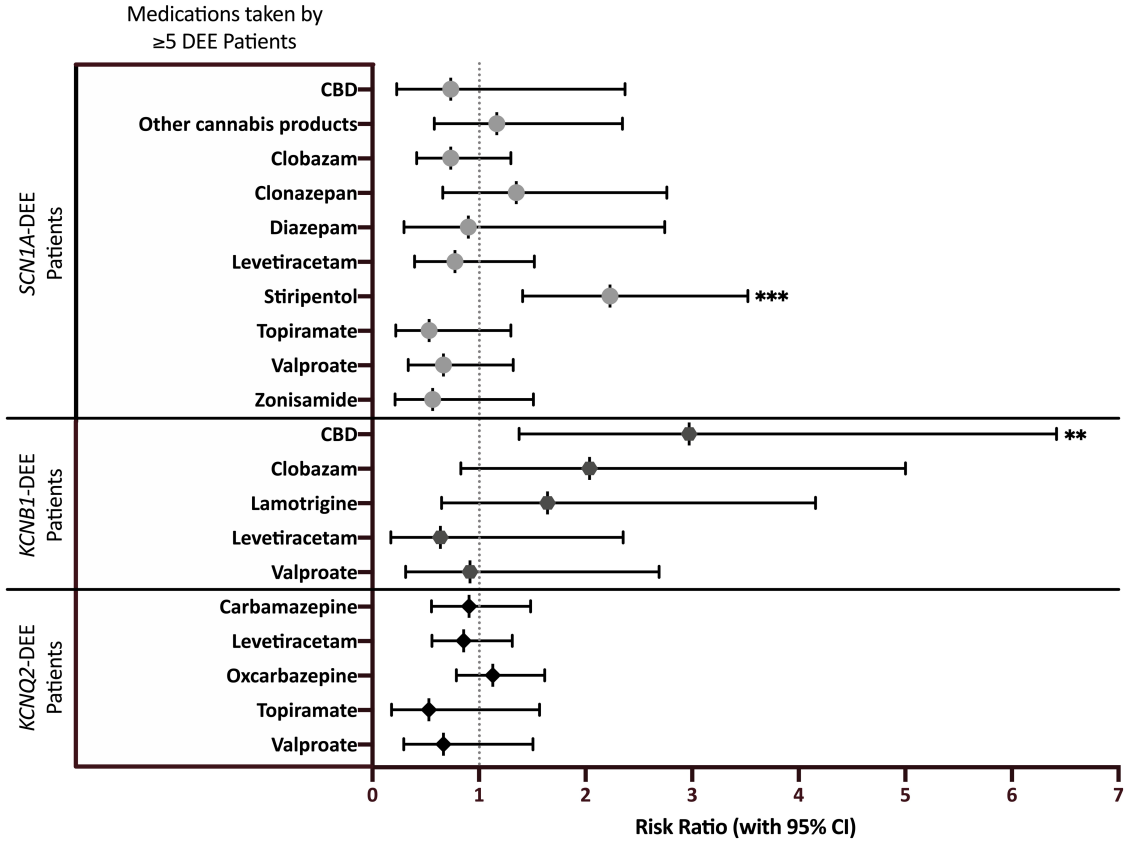

Medications taken by
≥5 DEE Patients
SCN1A-DEE Patients
CBD
Other cannabis products
Clobazam
Clonazepan
Diazepam
Levetiracetam
Stiripentol
***
Topiramate
Valproate
Zonisamide
KCNB1-DEE Patients
CBD
**
Clobazam
Lamotrigine
Levetiracetam
Valproate
KCNQ2-DEE Patients
Carbamazepine
Levetiracetam
Oxcarbazepine
Topiramate
Valproate
0
1
2
3
4
5
6
7
Risk Ratio (with 95% CI)

Figure 2.3: Incidence of GI symptoms in SCN1A-, KCNB1-, or KCNQ2-DEE patients taking antiseizure medications*. Relative risks ratios with 95% confidence intervals were calculated for (A) medications being taken by > 10 patients across all three disease cohorts as well as (B) medications being taken by ≥ five patients within each respective DEE cohort. Higher relative risk values are associated with an increased incidence of GI symptoms in patients currently taking a particular medication. ** = P < 0.01, *** = P < 0.001.*

2.5 Discussion

Children with ion channel-gene-linked DEEs have a high prevalence of GI symptoms, specifically constipation and dysmotility, which do not appear to be explained by common risk factors that frequently arise in the DEE population, such as limited functional mobility, anti-seizure medications, or presence of ASD. These symptoms create additional needs for medical care, place burdens on parents and caregivers, and negatively impact the quality of the patients' lives.

By initially focusing on DEE patients with variants in the genes encoding a subset of Na_v and K_v channels, we have documented the prevalence and risk factors for GI symptoms in channelopathy-linked DEE patients. Although we have no direct comparison to the frequency of similarly reported GI symptoms in a healthy population, a meta-analysis of 18 independent studies reporting on constipation in the general population found a median prevalence of constipation in children of ~9% (Interquartile Range: 5% to 17%), with the highest estimate being 30% (van den Berg, Benninga, & Di Lorenzo, 2006). These estimates from population-based studies are well below our overall finding of 55% (95% Confidence Interval: 47% to 62%) prevalence in DEE patients. Our findings emphasize the prevalence and severity of GI symptoms in channelopathy-associated DEE patients over a broad range of ages, while highlighting potential differences between three different channelopathy cohorts (Table 2.2).

While this study is focused on channelopathies, it is important to note that GI symptoms are prevalent in multiple DEEs. We further considered reports of GI symptoms from two non-channelopathy, gene-specific cohorts who had participated in a similar survey with the same questions regarding GI symptoms (in review). Parents of 71 *CHD2*- and *PACS1*-linked DEE patients reported rates of constipation and gastric dysmotility in their children, and the average prevalence of these symptoms across these two groups was 39%, which was lower than the average prevalence of GI symptoms, 55%, reported for the channelopathy groups (N = 159, consisting of *SCN1A*-, *KCNB1*-, and *KCNQ2*-linked DEE patients) ($P = 0.03$). Though relatively little is known about the role of the *CHD2* and *PACS1* genes in GI function, it is interesting to note that *CHD2* variants have been identified in multiple human gastric and colorectal cancers (M. S. Kim, Chung, Kang, Yoo, & Lee, 2011). Future research could determine if the GI symptoms in the *CHD2*- and *PACS1*-linked DEEs are similarly not explained by common risk factors often exhibited by DEE patients.

Risk factors for GI symptoms are common in the DEE population and include mobility limitations, ketogenic diet, and medications. None of these, however, appeared to be a major driving factor behind the prevalent GI symptoms in our DEE cohorts. For example, the *KCNQ2*-DEE patients were the least mobile group and exhibited the highest proportion of GI symptoms. Yet, within the *KCNQ2*-DEE cohort, there was no association between degree of mobility and GI symptoms. Further, there was no overall association between GI symptoms and mobility after adjusting for DEE type. Small, borderline associations were observed for well-known risk factors such as gastrostomy tube usage

and ketogenic diet therapy. As so few children had these interventions, their presence does not explain the high reported prevalence of GI symptoms in our study patients. Interestingly, sex was significantly associated with reported GI symptoms in *SCN1A*-, but not *KCNQ2*- or *KCNB1*-DEE patients; females with *SCN1A*-linked DEE were about three times as likely as their male counterparts to have these symptoms. Though females are more likely than males to develop chronic constipation in the general population (Johanson, Sonnenberg, & Koch, 1989), this differential was not apparent in the two other DEE groups. Independent replication of the finding is needed, but it could reflect sex-specific aspects of channelopathy pathophysiology.

Though previous research suggests a connection between bowel movements and seizure activity (Moezi et al., 2015), we observed no statistically significant association between GI symptoms and recent seizure activity in these channelopathy cohorts. In a separate part of the survey in which seizure triggers were queried, only a small percentage of parents reported bowel movements as a seizure trigger. Future studies could look more broadly at potential correlations between these symptoms in multiple forms of pediatric epilepsies.

Multiple anti-seizure medications are used in patients with DEEs, and these medications are known to have a wide range of side-effects, including GI dysfunction. While we are very cautious not to over-interpret our findings due to the small numbers, reporting use of any one medication and the multiple hypotheses tested, a few findings may inform new hypotheses to test formally in future studies. Stiripentol, a drug approved exclusively for

Dravet syndrome, was significantly associated with GI symptoms in the *SCN1A*-DEE group. GI symptoms were also correlated with cannabidiol (CBD) use in *KCNB1*-DEE patients only. Unlike stiripentol, CBD was taken by multiple children from all three DEE cohorts. Whether this particular finding for CBD reflects a specific effect on GI function in the context of $K_v2.1$ channel impairment remains to be seen.

The prevalence of GI symptomology in DEEs linked to variants in *SCN1A*, *KCNB1*, or *KCNQ2* is not well-explained by common risk factors for GI dysfunction and raises the question of whether the proteins encoded by these genes may contribute to proper GI tract function. *SCN1A* gene expression has been localized to the human colonic myenteric plexus, the branch of the enteric nervous system that controls bowel motility (Hetz et al., 2014). A study in mice found that mechanosensitive colonic fibers express $Na_v1.1$ (Osteen et al., 2016). While gastrointestinal expression of *KCNB1* and *KCNQ2* in humans is not well-characterized, $K_v2.1$ was found to be localized to murine colonic myenteric plexus (Seifi et al., 2014), and K_v7 channels have been localized to intrinsic neuronal populations as well as smooth muscle in the human GI tract (Peiris et al., 2017). Functionally, K_v7 channels are thought to be important for controlling contractile activity in the murine intestinal tract (Jepps et al., 2009) as well as mediating sensory colonic afferent responses to noxious stimuli in mice and humans (Peiris et al., 2017). Importantly, a wide variety of voltage-gated ion channel-encoding genes that are known to be expressed in the GI tract, in addition to brain, have been linked to various diseases, including other DEEs, that manifest with GI symptomology (Table III). GI symptoms in channelopathy-associated DEEs are common, serious, but poorly understood

comorbidities of these DEEs. The next steps will entail investigating the mechanisms and elucidating the roles of GI-expressed ion channels in GI function and dysfunction. The combination of our survey results and published data, suggest DEE-linked voltage-gated ion channel gene variants may also contribute to the GI dysmotility and constipation commonly reported in patients and lay the groundwork for future mechanistic studies in animal models of DEE.

Understanding the clinical condition is essential for guiding future investigations into the mechanisms by which variants in DEE-linked genes, particularly those encoding voltage-gated ion channels and other proteins with established links to GI function and dysfunction, may directly contribute to GI pathology via alterations in GI cell functionality or the development and activity of the enteric nervous system. As precision medicine becomes increasingly more feasible, gene-editing therapeutics could more directly elucidate the involvement of these proteins in specific DEE comorbidities, such as GI symptoms.

Several limitations must be acknowledged. First, the data presented in this study are derived from parent reports. This includes the diagnosis of specific ion channel variants linked to each child's DEE, although several parents provided genetic testing reports that corroborated the etiology. Because the families were recruited through groups that were specific to their child's disease, we expect any error in reporting for etiology to be minimal. In addition, the exact description of GI symptoms alone may not have been sufficiently specific and may have reflected how such questions are asked in a simple review-of-

systems often performed in a medical office. Additional questions regarding frequency, discomfort, and impact on appetite helped to define the scope of and contextualize the problem in the children's and parents' daily lives. Information about potential risk factors for GI symptoms was also obtained by report; however, the information was appropriate for obtaining in this manner. For functional mobility, we utilized the GMFCS, which has been previously validated for parent report (Morris et al., 2004). Other questions were developed in collaboration with parents and based on review of similar measures available throughout the literature. Finally, some questions were straightforward checklist items, such as whether a child had a gastrostomy tube or was being treated with the ketogenic diet. We also acknowledge that this survey was disseminated to an unknown number of people. We therefore cannot be certain that the responses of our 168 parent respondents can be extrapolated to the general population of children with DEEs. Notably, this is often the case in studies of rare diseases. The use of a web-based survey, however, did permit us to reach a large number of people, much greater than would have been possible in a single or even multi-center study over a relatively brief period of time. Some of our comparisons, particularly medications, were exploratory. We had the opportunity to ask these questions but often did not have the statistical power to address them. Further, the number of medications reported would reasonably raise concerns regarding multiple hypothesis testing. Thus, these results should be considered hypothesis-generating only. In this survey, we only directly asked about current use of medications for treatment of seizures and GI symptoms. We therefore do not have information on other medications with potential GI side-effects that may be used by children in this cohort for treatment of other DEE comorbidities.

In conclusion, this work provides novel information that highlights the importance of GI dysfunction in the DEEs and suggests these GI symptoms may be part of the disease itself and not just a result of poor mobility, medications, or other factors. Elucidating the role of ion channel dysfunction in GI symptoms for patients with DEE may guide future therapeutic efforts to alleviate this common and often distressing symptom.

Chapter 3 : Colonic Myenteric Neuron Dysfunction in a Mouse Model of *SCN1B*-linked Developmental and Epileptic Encephalopathy

Veronica Beck (VB) conducted the majority of data collection and all data analysis for Figure 3.1. **VB** contributed to data collection and performed the majority of data analysis for Figure 3.2. **VB** contributed to data collection and data analysis for Figure 3.3. **VB** completed all data collection and the majority of analysis for Figure 3.4, all data collection for Figure 3.5, and assisted with data analysis for Figure 3.6. Kathleen Ignatoski and Vinodh Balendran contributed to data collection for Figure 3.1D. Larissa Robinson-Cooper and Dennis Claflin contributed to data collection and analysis of Figure 3.2. Caroline Scheuing and Samantha Hodges contributed to data collection and analysis for Figure 3.3. Carter Dunaway performed data analysis for Figure 3.4D and all data analysis for Figure 3.5. Alberto Perez-Medina performed all data collection and the majority of data analysis for Figure 3.6. Experiments presented were performed in the laboratories of Susan Brooks, William Birdsong, and Lori Isom at the University of Michigan. Additional training was provided by Narayana Krishna Yelleswarapu in the laboratory of James Galligan at Michigan State University. **VB** and Lori Isom wrote this chapter.

3.1 Abstract

The enteric nervous system (ENS) is a complex neuronal network, located within the gastrointestinal (GI) tract, that controls the gut motility mechanisms necessary for proper digestion and excretion. GI motility is controlled by the myenteric plexus, which consists of neurons that communicate with GI smooth muscle cells to regulate contractions and relaxations. While the ENS is capable of functioning independently of central nervous system (CNS) input, mechanisms that control neuronal excitability and axonal pathfinding within the ENS are often analogous to those that govern neuronal excitability and connectivity in the CNS. Thus, it is not surprising that a growing body of clinical evidence shows that neurological diseases often present with comorbid abnormalities in the GI tract. Here, we investigate a potential mechanism underlying ENS dysfunction common to neurological diseases: altered activity of voltage-gated sodium channels (VGSCs) – which control the generation and propagation of action potentials in CNS and ENS neurons – and VGSC β1/β1B subunits, which modulate VGSC plasma membrane trafficking, gating, and voltage-dependence as well as participate inaxonal pathfinding and fasciculation.

VGSC gene variants, including variants in *SCN1B*, encoding the VGSC β1/β1B subunits, are linked to both developmental and epileptic encephalopathies (DEEs) as well as GI motility disorders. Furthermore, DEE patients often exhibit constipation, suggesting a connection between altered VGSC function and aberrant motility of the colon, resulting in

decreased rates of excretion of fecal material. The goal of this project was to gain insight into how loss-of-function (LOF) DEE variants in *SCN1B* contribute to colon dysmotility by studying the *Scn1b*-null mouse model of DEE. Our results show that *Scn1b*-null mice exhibit colonic dysmotility, which is driven by increasingly reduced and dysregulated colonic motility in line with disease progression. Furthermore, our data suggest that the mechanism driving this phenotype includes aberrant transcriptional regulation of voltage-gated ion channel genes that are necessary for proper enteric neuron excitability and signaling to effector SMCs. The results of this work may inform the mechanism of GI symptomology observed in *SCN1B*-linked DEE patients as well as in patients suffering from other VGSC-linked colonic motility disorders.

3.2 Introduction

Voltage-gated sodium channels (VGSCs), which control action potential initiation and propagation in excitable cells, are critical for normal activity of neurons (Beyder & Farrugia, 2012; Catterall, 2012; Chahine et al., 2008; Edokobi & Isom, 2018; O'Malley & Isom, 2015; J. Wang, Ou, & Wang, 2017). VGSCs consist of one pore-forming α subunit and two flanking β subunits (Catterall, 2000; Isom et al., 1992; Morgan et al., 2000; O'Malley & Isom, 2015; Yu et al., 2003). In the central nervous system (CNS) and peripheral nervous system (PNS), the VGSC β1 subunit and its soluble splice variant, β1B, – both encoded by *SCN1B* – modulate sodium and potassium current, chaperone VGSC α subunits to the plasma membrane, influence axon fasciculation and pathfinding, and regulate the transcription of VGSC and other voltage-gated ion channel (VGIC) genes (Aeby et al., 2019; Bouza et al., 2021; Brackenbury et al., 2010; Brackenbury et al., 2008; Brackenbury & Isom, 2011; Davis et al., 2004; Hull et al., 2020; Lopez-Santiago et al., 2011; Patino et al., 2009; Patino & Isom, 2010; Yuan et al., 2019). These previous studies illustrate the importance of VGSC β1 subunits in normal nervous system excitability and development; however, their role in a specific subset of PNS neurons – the enteric nervous system (ENS) located in the gastrointestinal (GI) tract – is unknown. Here, we examine the role of VGSC β1 subunits in ENS function as well as their effects on GI motility.

Variants in VGSC-encoding genes, including *SCN1B*, have been linked to numerous neurological diseases, including developmental and epileptic encephalopathy (DEE) and

generalized epilepsy with febrile seizures plus (GEFS+) (Aeby et al., 2019; Chahine et al., 2008; Kaplan et al., 2016; O'Malley & Isom, 2015). Notably, chronic or severe constipation is frequently reported in case studies of DEE patients with variants in VGSC-associated genes, suggesting impaired GI motility in these patients (Al-Mehmadi et al., 2016; Beck et al., 2021; Berecki et al., 2018; de Kovel et al., 2014; Gardella & Møller, 2019; Gay et al., 2008; Howell et al., 2015; Leipold et al., 2013; Villas et al., 2017; Ziobro et al., 2018). These clinical findings show that gene variants impacting VGSC function often produce both neurological and GI symptomology, predicting comorbid dysfunction of neurons in the CNS and ENS in VGSC-linked DEEs.

The mechanisms required for proper colon motility are largely controlled by the activity of the myenteric plexus, one of the two branches of the ENS, that is situated between the internal circular muscle and external longitudinal muscle layers of the intestinal wall (Furness et al., 2014; Hansen, 2003). This localized network of thousands of ganglia is interconnected by nerve fiber bundles, and the myenteric neurons also innervate interstitial pacemaker cells and effector smooth muscle cells (SMCs) in both the circular and longitudinal muscle layers to coordinate contractions and relaxations (Furness, 2000; Hansen, 2003; Iino & Horiguchi, 2006; Schneider et al., 2019).

VGSC α and β1/β1B subunit genes and/or their protein products are expressed by multiple excitable cell types in the mammalian GI tract, including SMCs, interstitial pacemaker cells, and myenteric neurons (Bartoo et al., 2005; Hetz et al., 2014; C. S. Hirst et al., 2015; Holm et al., 2002; O'Donnell et al., 2016; O'Donnell et al., 2019; Osorio et

al., 2014; Sage et al., 2007; Strege, Ou, et al., 2003). The VGSCs $Na_v1.5$ and $Na_v1.9$ – encoded by *Scn5a* and *Scn11a*, respectively – have been found to influence enteric neuron activity and subsequent GI motility patterns in mice (Copel et al., 2013; Osorio et al., 2014; Padilla et al., 2007). Likewise, variants in *SCN5A and SCN11A* have been linked to GI pathology in humans (Beyder et al., 2014; Neshatian et al., 2015; O'Donnell et al., 2016; Saito et al., 2009; Strege et al., 2018; Woods et al., 2015). VGSC β1 subunits are known to modulate the cell-surface expression and activity of $Na_v1.5$ and $Na_v1.9$ in mice; *Scn1b*-null cardiac myocytes exhibit increased $Na_v1.5$ expression and sodium current, and dorsal root ganglion (DRG) neurons exhibit decreased cell-surface $Na_v1.9$ expression (Brackenbury et al., 2010; Lin et al., 2015; Lopez-Santiago et al., 2011; Lopez-Santiago et al., 2007). Furthermore, a recent study found reduced *SCN1B* mRNA levels in in both ganglionic and aganglionic portions of colon in patients with Hirschsprung's disease, a GI disorder characterized by atypical ENS development and subsequent colonic dysmotility (O'Donnell et al., 2019). These data support a role of VGSC β1 subunits in the constipation experienced by VGSC-linked DEE patients.

To determine the contribution of *SCN1B* to the regulation of ENS activity and development in colon, we analyzed colonic motility patterns as well as myenteric plexus structure, activity, and VGIC gene expression in *Scn1b*-null mice, a mouse model of *SCN1B*-linked DEE (DEE52). We observed colonic dysmotility in *Scn1b*-null mice, mirroring the constipation observed in DEE patients. Functional experiments revealed irregular stretch-induced colonic contractions in *Scn1b*-null mice, likely driven by uncoordinated activity of myenteric neurons. We found myenteric neuron size, density,

and subtype ratios to be normal in *Scn1b*-null mice; furthermore, stimulus-induced signaling from myenteric neurons to SMCs was intact. *Scn1b*-null mice exhibited aberrant inter-ganglionic connectivity as well as age-dependent alterations in VGIC gene expression, which may explain the dysmotility. This work predicts multiple roles for VGSC β1/β1B subunits in ENS and suggests how loss-of-function (LOF) *SCN1B* variants may contribute to the GI dysfunction observed in DEE52 patients as well as in patients suffering from other VGSC-linked DEEs.

3.3 Methods

Animals

Scn1b-null and WT mice were generated from *Scn1b*$^{+/-}$ mice as described (Chen et al., 2004). *Scn1b*$^{+/-}$ mice were maintained on the C57BL/6J background for over 20 backcrossed generations and are thus congenic. To generate *Scn1b*-null and WT mice with the Chat gene labeled with an endogenous green fluorescent protein (GFP) epitope tag. *Scn1b*$^{+/-}$ mice were crossed with ChAT−eGFP mice that express eGFP in choline acetyltransferase (ChAT)-expressing neurons, received from Dr. G. Richerson at the University of Iowa (Y. Wu et al., 2019). Male and female animals ages postnatal day (P) 13-17 were used in all experiments. Animals were housed in the Unit for Laboratory Animal Medicine at the University of Michigan. All procedures adhered to NIH guidelines and were approved by the University of Michigan Institutional Animal Care and Use Committee.

Table 3.1: Primary and secondary antibodies used in Chapter 3 experiments

Primary Antibody	***Host***	***Dilution***	***Source***	***Catalog #***
ANNA-1(HuC/D)	Human	1:10,000	Human anti-sera (Vanda Lennon, Mayo Clinic, Rochester, MN)	
nNOS	Rabbit	1:200	ThermoFisher Scientific	61-7000
Tubulin β3 (clone TUJ1)	Mouse	1:200	BioLegend	801202

Secondary Antibody	***Dilution***	***Source***	***Catalog #***
AlexaFlour® goat anti-human 594 nm	1:500	ThermoFisher Scientific	A-11014
AlexaFlour® goat anti-rabbit 647nm	1:500	ThermoFisher Scientific	A-32733
AlexaFlour® goat anti-mouse 568 nm	1:500	ThermoFisher Scientific	A-11004

Table 3.2: Drugs used in Chapter 3 experiments

Drug	***Concentration used***	***Source***	***Catalog #***	***Mechanism***
Bethanechol Chloride	10 μM	Sigma-Aldrich	1071009	Muscarinic acetylcholine receptor agonist
Sodium Nitroprusside	5 μM	Sigma-Aldrich	71778	Nitric oxide donor
Tetrodotoxin citrate	300 nM	Alamone	T-550	Selective sodium channel blocker
MRS 2179	10 μM	Tocris	0900	Selective $P2Y_1$ receptor antagonist
Mecamylamine Hydrochloride	10 μM	USP	137600660H154	Nicotinic acetylcholine receptor antagonist
Scopolamine Hydrobromide	1 μM	Sigma-Aldrich	SLBX2802	Muscarinic acetylcholine receptor antagonist
Nitro-L-Arginine	100 μM	Sigma-Aldrich	N5501-16	Nitric oxide synthase inhibitor
Nifedipine	1 μM	Sigma-Aldrich	N7634	L-type calcium channel antagonist

Behavioral video observation

Two age-matched, genotype-matched littermates were observed in a static housing cage with gel chow (details) for 35 minutes. In each pairing, one mouse was marked with tape to differentiate individuals for future analysis. Videos were recorded using OmniPlex D software and hardware acquired from Plexon Inc. in Dallas, Texas, USA. Cameras were

oriented to provide full field of view of the chow. Each video was observed and analyzed manually. “Feeding” was defined as licking, biting, or chewing the gel food. Total fecal pellets expelled during the observation period were counted at the end of the 35-minute observation period; additionally, total fecal pellets observed at the bottom of the observation cage were counted at the end of the experiment.

Gastric bead assay

To assess colon motility, we inserted a glass bead (1.2 mm in diameter) ~1 cm into the distal colon of *Scn1b*-null and WT mice using a lubricated pipette tip and timed bead expulsion.

Immunohistochemistry tissue preparation

Mice were anesthetized using isofluorane then euthanized via decapitation, and distal colon was dissected into 4°C 1X Krebs solution (117 mM NaCl, 4.7mM KCl, 2.5 mM CaCl2, 1.2 mM MgCl2, 1.2 mM NaH2PO4, 25 mM NaHCO3, and 11 mM dextrose) and flushed of fecal contents. The colon tissue was then prepared for cryosections or whole mounts.

Cryosections: Dissected colon tissue was fixed for two hours in 4% paraformaldehyde at room temperature, washed 3 times for 10 minutes with 1X phosphate-buffered saline (PBS) then cryoprotected in 30% sucrose overnight at 4°C. Tissues were then embedded in O.C.T. (Tissue-Tek), and 8 µm sections were cut on a Leica cryostat and stored at −20 °C until processing for immunohistochemistry. For distal muscle thickness analysis, mounted on slides using ProLong Gold anti-fade reagent with DAPI (Life Technologies), cover slipped, and stored in at 4°C until imaging. For distal

colon circumference and muscle area measurements, hematoxylin and eosin staining of cyrosections was performed by the University of Michigan Rogel Cancer Center Tissue and Molecular Pathology Shared Resource.

Whole mounts: Dissected distal colon tissue was pinned to a Sylguard silicone coated petri dish with mucosa facing up and fixed overnight in 4% paraformaldehyde at 4°C. The tissue was then washed 3 times for 10 minutes with 0.1M phosphate buffer (PB) solution. Whole-mount longitudinal muscle myenteric plexus preparations (LMMPs) were achieved via removal of the mucosa, submucosal plexus, and circular muscle layers of the tissues by microdissection. The samples were then bathed in blocking buffer (4% goat serum, 0.4% Triton X-100 in 0.1% PB) for one hour and then incubated with primary antibodies (diluted in blocking buffer) overnight at 4°C and washed 3 times for 10 minutes with 0.1M PB. From this point all steps were performed in the dark to minimize photobleaching of secondary antibodies. Samples were incubated with secondary antibodies (diluted in blocking buffer) for 2 hours, washed 3 times for 10 min in 0.1M PB, then mounted on slides using ProLong Gold anti-fade reagent with DAPI (Life Technologies), cover slipped, and stored in at 4°C until imaging.

Confocal microscopy and analysis

Slides were imaged on a Zeiss LSM 880, located in the University of Michigan Department of Pharmacology, with a 20X objective or 63X oil objective, and excitation was accomplished using 405, 488, 561, and 633 nm lasers. Images consisted of maximum intensity projections of z-stacks (optical section width automatically calculated by Zeiss Zen software based on tissue thickness and channel quantity: 20X images were acquired

at 1.26 μm intervals for sections with three layers of fluorescence and at 1.07 μm intervals for sections with four layers of fluorescence, while 63X images were acquired at 0.44 μm intervals for sections with three layers of fluorescence and at 0.43 μm intervals for sections with four layers of fluorescence. Images were analyzed manually using Zeiss Zen software and ImageJ. Unless otherwise specified, 4 to 10 randomly selected fields per sample were analyzed for all quantifications.

Quantitative analysis of enteric neuron density and size: Using whole mount-stained samples, we counted the number of enteric neurons (ANNA-1$^+$ cells) to determine density. Enteric neuron subtype proportions were quantified by counting the number of ANNA-1$^+$, eGFP$^+$ (indicating ChAT$^+$ cells), and nNOS$^+$ cells. For size analysis, the FIJI StarDist plugin (Schmidt, Weigert, Broaddus, & Myers, 2018) was utilized to automatically measure soma area. Imaging and analysis were performed blind to genotype.

Quantitative analysis of axonal networks. Clusters of neurons labeled with nNOS, TUJ1, or ChAT-eGFP were labeled as distinct ganglia on ImageJ. Connections between two ganglia were deemed primary axonal connections. Axonal connection length was measured by drawing a segmented line between each axon's ganglia-contact points. Width was measured at the middle-most point of each axonal connection. Any distortions in the image were excised to provide a more accurate area measurement. Additionally, within a single image the ChAT-eGFP fluorescence layer was often merged with the TUJ1 or nNOS fluorescence layer to better visualize connections.

Organ bath

Dissected distal colon segments were flushed with 1X Krebs solution and mounted with 10-0 monofilament nylon ligatures on both ends between a stationary attachment point

and an isometric force transducer (Aurora Scientific, model 400A). The chamber was then mounted on the stage of an inverted microscope (Zeiss Axiovert 100) and perfused with 37°C 1X Krebs solution at a flow rate of 3 solution changes/min. A resting tension of 0.2 grams was applied to each tissue preparation. Baseline contraction patterns were recorded over 30 minutes. Electrical stimulation was delivered via platinum plate electrodes placed on either side of and parallel to the colon segment. The electrical stimulation paradigm consisted of a 20 Hz train of pulses, 0.5 ms pulse duration, with train durations of 500 ms. Stimulus intensity was 40-70mA, controlled using a high-power stimulator (Aurora Scientific, model 701C) operating in constant-current mode. All drug concentrations listed in Table 6. Bethanechol chloride and sodium nitroprusside (SNP) were added into each bath sequentially to produce maximal contraction and relaxation responses, respectively. The tissue was then stimulated using an electrode at increasing intensities until a stimulation intensity was reached that elicited reproducible relaxation and contraction responses from the tissue. Tetrodotoxin (TTX) was then added to block neuromuscular transmission in order to reveal myogenic responses and ensure that the chosen intensity of the transmural electrical stimulus was specifically exciting enteric neurons in the distal colon segment. The colon segments were repeatedly stimulated during the TTX condition to ensure that the contraction events that emerged in the presence of TTX were not neuronally mediated. MRS 2179 was administered to block purinergic-mediated muscle relaxation to reveal the nitrergic component of relaxation, mecamylamine was administered to block cholinergic signaling to myenteric neurons, scopolamine was administered to block cholinergic signaling to smooth muscle, and nitro-L-arginine (NLA) was administered to block nitrergic-mediated muscle relaxation to reveal

the purinergic component of relaxation. Raw force traces were acquired using a digital oscilloscope (Teledyne-LeCroy, model HDO6034A).

Quantitative analysis of tissue contractions and relaxations. Traces were analyzed off-line using digital signal processing software (Signo, Alameda Applied Sciences). To distinguish distension-induced peristaltic contractions from intrinsic myogenic patterns, we only analyzed contraction events that measured at least twice the amplitude of a tissue's baseline tension.

Intracellular recordings of excitatory and inhibitory junction potentials at the circular smooth muscle

Dissected distal colon tissue was pinned to a Sylguard silicone-coated petri dish with mucosa facing up, and muscosal and submucosal layers were then removed by microdissection to expose the circular smooth muscle layer. A 0.5 cm^2 section was then transferred to a 5 ml silicone elastomer-lined recording chamber. Tissues were gently stretched and pinned to the recording chamber with small stainless-steel pins. The chamber was then mounted on the stage of an inverted microscope and perfused with 37ºC 1X Krebs solution at a flow rate of 4 ml/min. To limit spontaneous muscle contractions during intracellular recordings, the L-type VGCC antagonist nifedipine was added to the perfusing Krebs solution for the duration of each experiment. The preparations were allowed to acclimate for 30 minutes. Borosilicate 1.0 mm x 0.5 mm ID w/fiber glass (FHC Inc., Bowdoin, ME) microelectrodes were filled with 2M KCl (tip resistance, 60-120 MΩ) and were then used to impale circular smooth muscle cells. Focal stimulation was performed using a FHC tungsten metal bipolar electrode (2-3 mΩ) in the recording chamber. The electrodes were connected to a Grass S88 stimulator and

stimulus isolator constant current unit (Grass Technologies, West Warwick, RI). The electrical stimulation paradigm consisted of a 10 Hz train, 0.5 ms pulse duration, 60 V intensity with train durations of 300 ms. Focal stimulation following co-application of MRS 2179 (10 μM) and NLA (100 μM) to block inhibitory myenteric neuron signaling abolished inhibitory junction potentials and unmasked excitatory junction potentials. All membrane potential recordings were obtained using a Multiclamp 700b amplifier (Molecular devices, San Jose, CA), a Digidata 1550B analog-to-digital signal converter (Molecular devices, San Jose, CA), and Axoscope 11.1 software (Molecular Devices, San Jose, CA). The amplified signal was sampled at 2 kHz and filtered at 1 kHz. Successful impalements of a circular smooth muscle cell were determined by a rapid drop in the resting membrane potential, and only cells that sustained a resting membrane potential bellow -40 mV were considered for statistical analysis. Mean average values were determined by averaging the total number of cells recorded from all animals per treatment.

RT-qPCR

Dissected distal colon tissue was pinned to a Sylguard silicone-coated petri dish with mucosa facing up and fixed overnight in 4% paraformaldehyde at 4°C. The tissue was then washed 3 times for 10 minutes with 0.1M phosphate buffer (PB) solution. The mucosa and submucosal plexus were then removed by microdissection, leaving the circular and longitudinal muscle layers and myenteric plexus. Tissues from two mice of same age and genotype were pooled and placed in guanidine thiocyanate lysis-containing buffer, homogenized mechanically with a tissue homogenizer, lysed through a sterile, 22-gauge hypodermic needle, and ultrasonically sonicated before RNA isolation. RNA was isolated from the distal colon samples using the Qiagen RNeasy Fibrous Tissue

Mini kit according to the manufacturer's instructions. Isolated RNA was analyzed on a NanoDrop One Spectrophotometer (ThermoFisher Scientific) to ensure adequate concentration and purity and then stored at -80°C until use. cDNA was generated from 0.5 µg of RNA using Reverse Transcriptase SuperScript III (RT SS III), random primers (Invitrogen), and dNTPs (Invitrogen). Random primers, dNTPs, and RNA were incubated at 65°C for 5 minutes. Salt buffers, 0.1M DTT, RNaseOUT, and RT SS III, were added and incubated at 25°C for 5 minutes, 50°C for 60 minutes, and then at 70°C for 15 minutes. cDNA was either kept undiluted or diluted 1:3 in water. Comparative qPCR using SYBR Green (Applied Biosystems) and gene-specific primers (Integrated DNA Technologies) was performed on a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems). ΔΔCt values were calculated by comparing genes of interest with the endogenous control gene GAPDH and normalizing to the WT control condition to determine comparative gene expression.

Statistics

No more than two measurements were acquired from any one animal for each experiment. Data points for each experiment are presented as mean measurement per animal ± SEM of mean measurements from all animals. Statistical significance (*P* value less than 0.05) of comparisons between genotypes was determined using Student's *t* test.

3.4 Results

Scn1b-null mice exhibit colonic dysmotility

The phenotype of *Scn1b*-null mice, including spontaneous and generalized seizures as well as failure-to-thrive (FTT) by post-natal day (P) 13, ataxia, and premature death around ~P17-P19 (Chen et al., 2004; Yuan et al., 2019), mirrors the intractable seizures, developmental delay, ataxia, and high risk for sudden unexpected death in epilepsy (SUDEP) observed in DEE52 patients (Aeby et al., 2019; Ogiwara et al., 2012; Patino et al., 2009; Personal_Communication, 2019 & 2020; Ramadan et al., 2017). Similar to other DEE patients (Beck et al., 2021), DEE52 patients are also reported to experience GI symptoms, including constipation (Personal_Communication, 2019 & 2020). To determine if *Scn1b*-null mice also exhibit constipation, we recorded pairs of null and wild-type (WT) littermates in observation chambers for a period of 35 minutes. Despite significantly increased feeding behavior (P = 0.015; Figure 3.1A), *Scn1b*-null mice exhibited a reduction in ad libitum defecation events both during (P = 0.0668; Figure 3.1B) and outside the time range of the observation period (P = 0.0013; Figure 1C). To more directly test colonic motility, we inserted a glass bead into the distal colon of *Scn1b*-null and WT mice and recorded time to bead expulsion through the rectum. *Scn1b*-null mice exhibited a significant delay in bead expulsion (P = 0.0028; Figure 3.1D), further confirming the constipation phenotype in these animals. Because the *Scn1b*-null phenotype includes FTT, predicting potential deficits in nutrient absorption, we proposed that a reduction in colonic smooth muscle may contribute to the colonic dysmotility;

however, we observed no differences in multiple metrics of musculature between *Scn1b*-null and WT distal colon cyrosections (Figures 3.1E-1H).

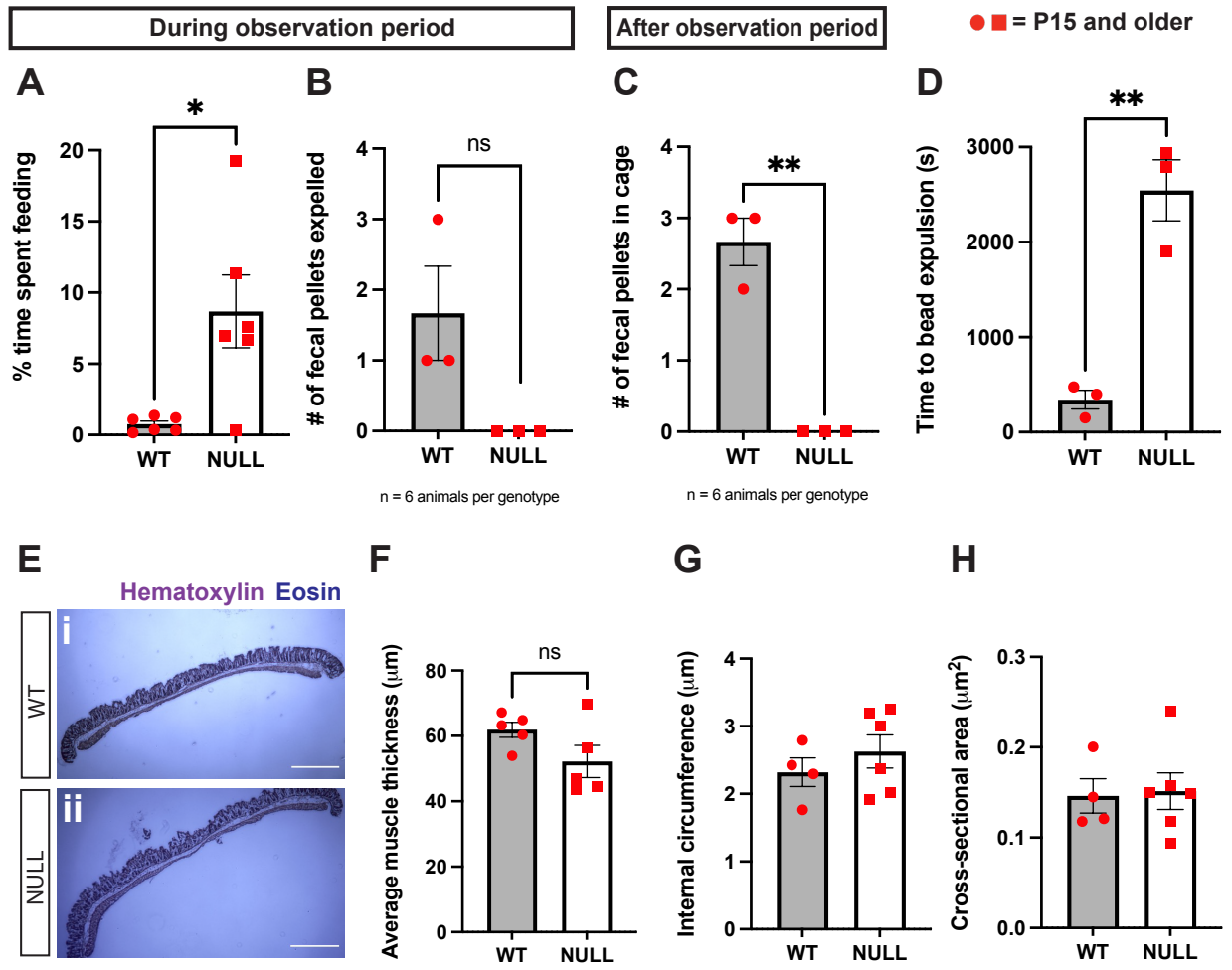


Figure 3.1: Scn1b-null mice exhibit constipation and colonic dysmotility despite increased feeding behavior and normal distal colon muscle size. *We observe that P15+ Scn1b-null mice, despite A) increased time spent feeding during a 35-minute observation period (P = 0.015), exhibit decreased ad libitum defecation events B) during the defined observation period (P = 0.0668) as well as C) throughout the entire time spent in the observation cage (P = 0.0013). D) P15+ Scn1b-null mice took significantly longer to expel a 1.5mm bead inserted ~1cm into distal colon (P = 0.0028). E i-ii) this constipation phenotype is not due to decreased musculature in P15+ Scn1b-null distal colons compared to age-matched WT mice, as measured by F) average smooth muscle thickness (P = 0.1122), G) average internal circumference of distal colon segments, and H) average cross-sectional area of distal colon muscle sections. Scale bars: 0.5 mm. * = P < 0.05, ** = P < 0.01. Data are represented as the mean ± SEM. B,C) Each data point represents the cumulative fecal output from two age-matched, genotype-matched animals observed in the same cage.*

Scn1b-null mice have dysregulated neurogenic colonic motility patterns

Proper colonic motility relies on functional neurotransmission from colonic myenteric motor neurons to effector SMCs. Inhibitory motor neurons release nitric oxide (NO) to induce smooth muscle relaxation, while excitatory motor neurons induce smooth muscle contraction via acetylcholine (ACh) signaling (Furness, 2000; Greig & Cowles, 2017; Hansen, 2003; Qu et al., 2008; Schneider et al., 2019). To better understand the mechanisms underlying the *in vivo* colonic dysmotility in *Scn1b*-null mice, the distal colon was dissected from null or WT mice and placed in a temperature-controlled bath of oxygenated Krebs solution. The colon was longitudinally stretched to an initial tension of 0.2 grams, and the resulting motility patterns were recorded in the absence of drugs or in the presence of: sodium nitroprusside (SNP; 5 μM), a NO donor that induces a maximum relaxation response; bethanechol chloride (10 μM) a SMC ACh receptor agonist that induces a maximum contraction response, or tetrodotoxin (TTX, 300 nM), a selective VGSC blocker that silences neuronal activity.

We observed no difference in the SNP-induced maximum relaxation responses (Figure 3.2A), or bethanechol chloride-induced maximum contraction responses (Figure 3.2B) between genotypes, indicating that SMCs in *Scn1b*-null colon are able to function properly in response to exogenous inhibitory and simulated excitatory neurotransmission paradigms. We applied focal stimulation to *Scn1b*-null and WT tissues to confirm neurogenic relaxation and contraction responses (Figure 3.2Ci/ii). We then analyzed the magnitude, frequency, and regularity of contractions in the absence and presence of 300 nM TTX to specifically block TTX-S VGSCs. Of note, two *Scn1b*-null mice, aged P14 and

P16, respectively, exhibited no stretch-induced contraction events. In the preparations in which we observed a contractile response, we observed no genotypic differences in the average magnitude of distension-induced baseline contractions in the absence or presence of TTX (Figure 3.2E,G), suggesting that *Scn1b*-null smooth muscle contractility is intact and unaffected by TTX addition. We further validated that the chosen TTX concentration was specifically blocking endogenous neurogenic signaling to SMCs by repeatedly performing focal stimulation (at the same intensities at applied in Figure 3.2C) and observing no immediate relaxation or contraction responses. Though baseline contraction magnitude was no different between *Scn1b*-null and WT mice, we observed substantial age-dependent differences in contraction frequency and regularity the *Scn1b*-null animals compared to WTs. *Scn1b*-null mice aged P15 - P16 showed a significant reduction in the average number of contractions per minute compared to P14-P16 WTs under baseline conditions (P = 0.0036; Figure 3.2Di/ii, E). This aberrant *Scn1b*-null contraction frequency was abolished following perfusion of TTX (P = 0.0104; Figure 3.2D iii/iv, H), suggesting that aberrant activity of *Scn1b*-null myenteric neurons underlies the observed reduction in contraction events compared to WT animals. P15+ *Scn1b*-null mice also exhibited significantly increased time between individual contraction events [or inter-spike intervals (ISIs)] (P = 0.0176; Figure 3.2Di/ii, I) as well as significantly increased standard deviation (SD) of these ISIs (P = 0.0289; Figure 3.2Di/ii, J). Both of these genotype-specific differences were ablated in the presence of TTX (Figure 3.2Fii/ii, I, J), once again suggesting aberrant enteric neuronal activity was driving the irregular contraction patterns. Both WT and *Scn1b*-null tissues stimulated before TTX addition to establish baseline neuronally mediated contraction capacity, were repeatedly stimulated

under TTX conditions to verify that neurons were not active (Diii,iv), and stimulated again after TTX was washed from the system to verify that the neurons regained normal excitability. The P16 *Scn1b*-null animal that showed no contraction events at baseline also exhibited no contractions under TTX conditions, but this animal did exhibit contractions in response to focal stimulations both before TTX perfusion and after TTX wash. The P14 *Scn1b*-null animal that showed no contraction events at baseline, did exhibit contractions in the presence of TTX as well as in response to focal stimulations before and after TTX administration. Strikingly, none of these observed differences in baseline contraction frequency and/or regularity were observed in P14 *Scn1b*-null animals, which did not differ from P14-P16 WTs in contractions per minute (Figure 3.2K), ISI (Figure 3.2L), or ISI SD measures (Figure 3.2M). Taken together, these data show that *Scn1b*-null mice have reduced and irregular colonic contraction patterns that are developmentally regulated and dependent on myenteric neuron activity.

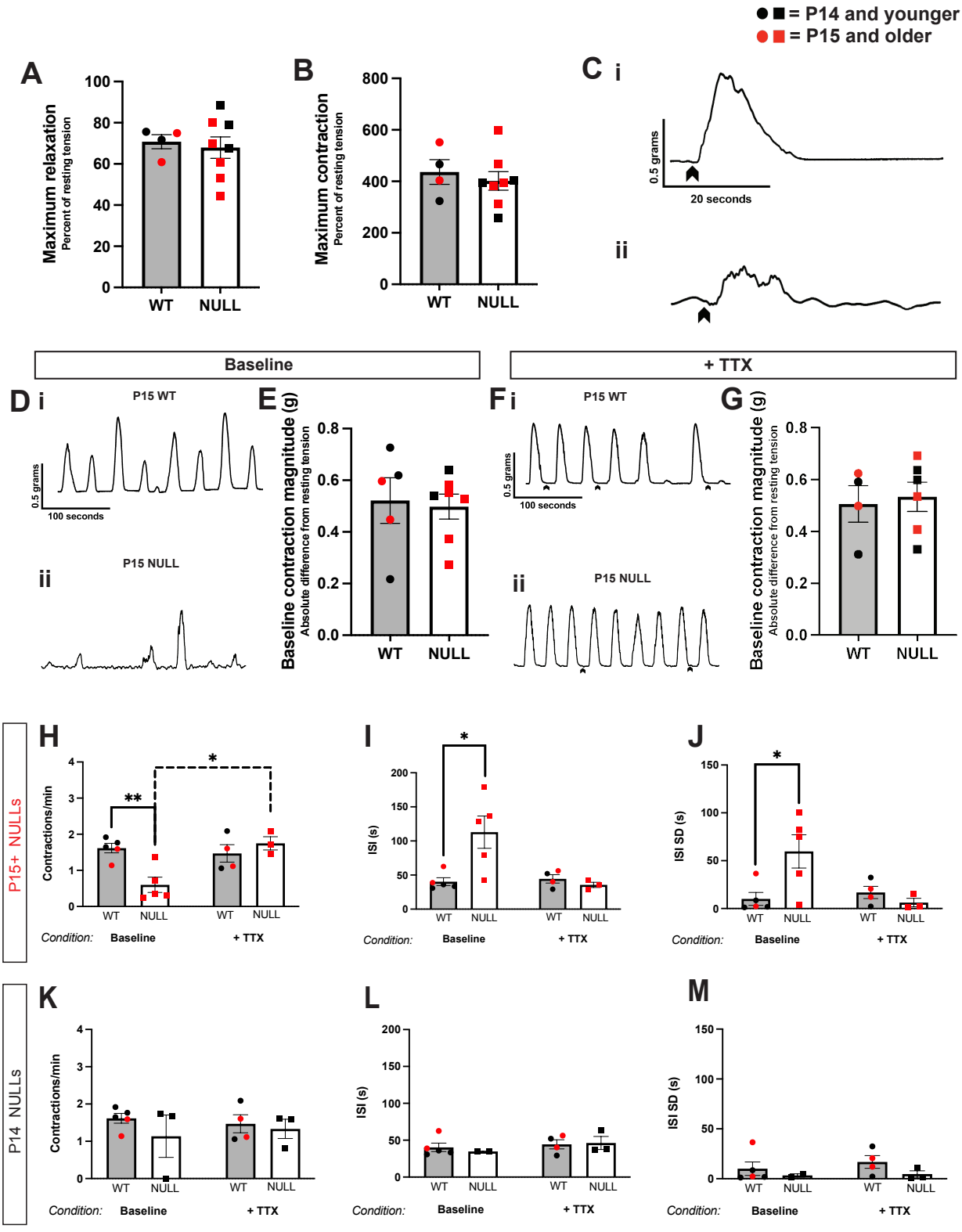


Figure 3.2: Scn1b-null mice have normal contraction and relaxation amplitudes in distal colon but exhibit age-associated diminished and irregular neuronally mediated motility patterns. *Organ bath recordings were performed on longitudinally*

*stretched segments of distal colon. We observed no difference in the relative responses to A) exogenous inhibitory neurotransmission or B) simulated excitatory neurotransmission. C) Focal stimulation (black arrows) induced neurogenic relaxation and contraction responses in both Scn1b-null and WT animals. (Fi/ii) Addition of 300nM TTX blocked neurogenic responses to focal stimulation (black arrows). (E,G) While there was no difference in mean distension-induced peristaltic contraction magnitude between genotypes in either baseline or TTX conditions, we did observe differences in baseline contractility patterns between WT (Di) and Scn1b-null (Dii) mice. P15+ Scn1b-null exhibit H) significantly decreased baseline contraction frequency (P = 0.0036) as well as I) significantly increased time between contractions [ISIs] (P = 0.0104) and J) standard deviation of ISIs (P = 0.0289). All of these genotype-dependent differences were abolished when neuronal activity was blocked in the presence of TTX (Fi/ii,H, I, J). P14 Scn1b-null animals exhibited no difference from P14-P16 WT contractility patterns in the baseline or TTX conditions (K-M). * = P < 0.05, ** = P < 0.01. Data are represented as the mean ± SEM.*

Scn1b-null mice show age-associated alterations in VGIC-encoding gene expression

Recent work has shown that the cleaved intracellular domain of the β1 subunit can translocate to the nucleus where it regulates transcription of VGSC and other VGIC genes (Bouza et al., 2021). *Scn1b*-null mice exhibit altered protein and gene expression of VGICs in brain, spinal cord, and heart (Bouza et al., 2021; Brackenbury et al., 2010; Chen et al., 2004; Lin et al., 2015; Lopez-Santiago et al., 2011; Lopez-Santiago et al., 2007). Multiple types of VGICs are expressed in the mammalian ENS and GI tract. The VGSCs $Na_v1.5$ (*Scn5a*) and $Na_v1.9$ (*Scn11a*) are thought to be important in enteric neuron and SMC excitability (Copel et al., 2013; O'Donnell et al., 2016; Osorio et al., 2014; Padilla et al., 2007). Voltage-gated calcium channels (VGCCs), specifically $Ca_v2.2$ (*Cacna1b*) and $Ca_v2.3$ (*Cacna1e*), are thought to control neurotransmitter release of ACh and NO, respectively (Bian et al., 2004; Bridgewater et al., 1995; E. Rodriguez-Tapia, Perez-Medina, Bian, & Galligan, 2016; E. S. Rodriguez-Tapia et al., 2017; Rugiero et al., 2002; Shuttleworth & Smith, 1999; Smith et al., 2003; Vogalis et al., 2002; Wessler et al., 1990). We dissected distal colon sections containing longitudinal muscle, circular muscle, and myenteric plexus from age-matched P14 and P16 *Scn1b*-null and WT mice. We then isolated RNA from these samples and performed qRT-PCR to determine whether differences in VGIC gene expression may explain the colonic dysmotility observed in P15+ *Scn1b*-null animals. We observed significant increases in the expression of *Scn9a* (P = 0.0041; Figure 3.3A) and *Scn11a* (P = 0.0058; Figure 3.3A) in P16 *Scn1b*-null compared to P16 WT samples. Additionally, we observed significant decreases in the VGCC genes *Cacna1b* (P = 0.0026; Figure 3.3B) and *Cacna1e* (P = 0.0135; Figure 3.3B) in P16 *Scn1b*-null animals compared to age-matched controls. In contrast, we observed

no significant differences in the relative expression of any VGIC genes in the P14 *Scn1b*-null animals compared to P14 WT samples.

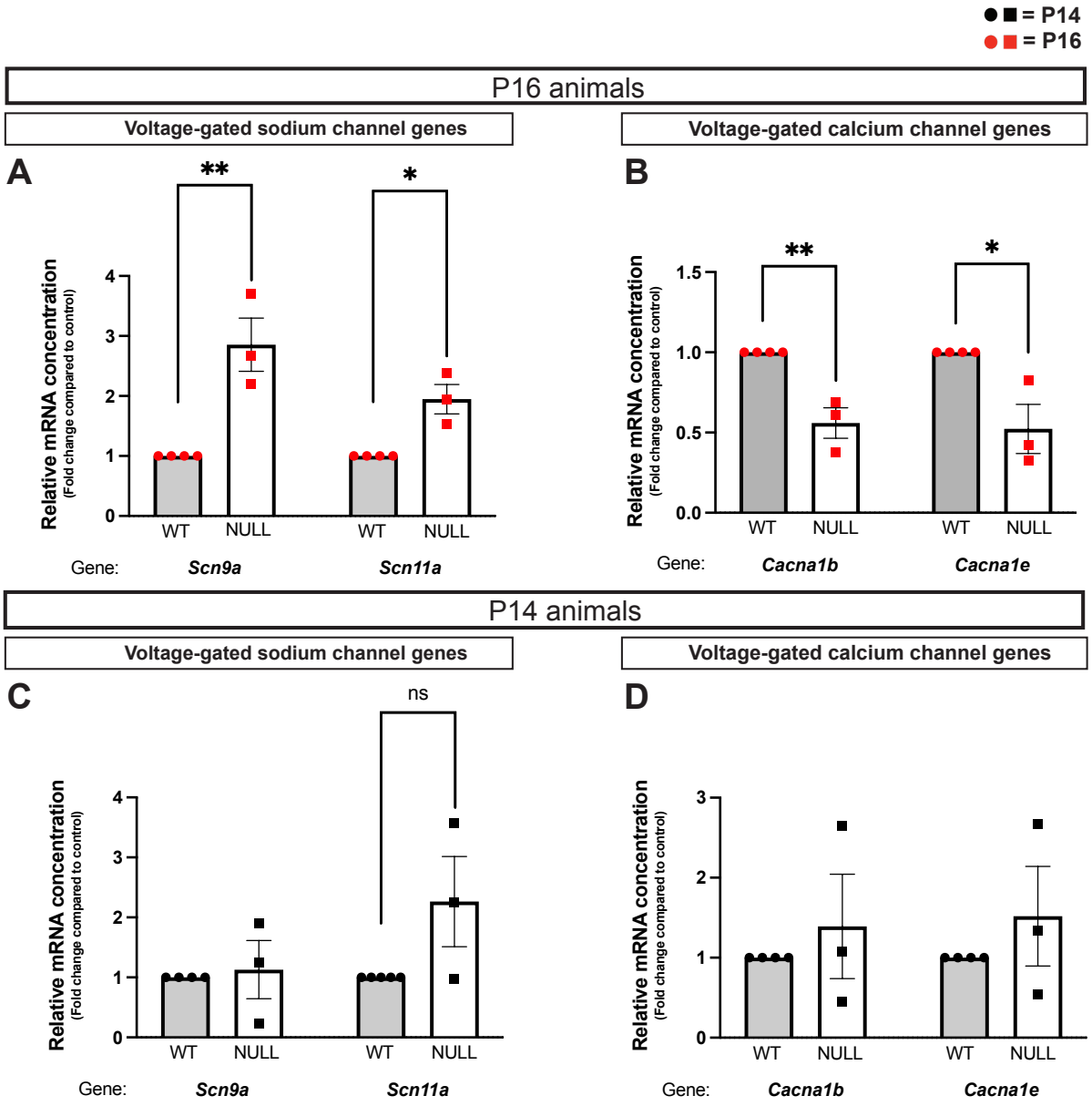


Figure 3.3: Scn1b-null mice exhibit age-dependent altered expression of VGSC and VGCC-encoding genes. *RT-qPCR performed on smooth muscle + myenteric plexus preparations from P14 and P16 Scn1b-null and WT mice reveal A) increased expression of VGSC-encoding genes, Scn9a (P = 0.0041) and Scn11a (P = 0.0058), and B) decreased expression of VGCC-encoding genes, Cacna1b (P = 0.0026) and Cacna1e (P = 0.0135), in P16 Scn1b-null animals. C, D) None of these changes in VGIC gene expression – including Scn9a (P = 0.1004) – are observed in P14 Scn1b-null animals. * = P < 0.05, ** = P < 0.01. Data are represented as the mean ± SEM.*

Scn1b-null mice have normal density, size, and ratios of myenteric motor neurons

Altered *SCN1B/Scn1b* expression has been linked to deficits in enteric neuron expression in human colon (O'Donnell et al., 2019) as well as aberrant neuronal migration and proliferation in mouse CNS (Brackenbury et al., 2013). Therefore, we investigated whether P14 (black) and/or P15+ (red) (Figure 3.4) *Scn1b*-null mice had normal numbers and somal sizes of excitatory and inhibitory myenteric neurons in distal colon, as altered myenteric motor neuron density or development of excitatory and/or inhibitory enteric myenteric motor neuron populations may contribute to the observed colonic dysmotility. To image myenteric neurons, we extracted distal colon from age-matched *Scn1b*-null and WT ChAT-eGFP mice, in which the enzyme choline acetyltransferase (ChAT), which synthesizes the primary excitatory enteric neurotransmitter ACh, is labeled with an endogenous GFP tag. After the colons were dissected, we stripped away the mucosa, submucosa, and circular muscle layers to reveal the myenteric plexus. These LMMPs were then immunostained with antibodies against ANNA-1 (HuC/D antisera), which labels both excitatory and inhibitory enteric neurons (Kulkarni et al., 2017), as well as neuronal nitric oxide synthase (nNOS), which labels inhibitory enteric neurons. We observed normal neuronal and ganglionic densities (Figures 3.4C ,3.4D) as well as normal mean neuron size (Figure 3.4E) in the distal colons of *Scn1b*-null animals. We then analyzed the subpopulations of excitatory ($ChAT^+$) and inhibitory ($nNOS^+$) enteric neurons and observed no differences between genotypes in excitatory neuron proportion or mean size (Figures 3.4F, 3.4G) or inhibitory neuron proportion or mean size (Figures 3.4H ,3.4I). Furthermore, there were no age-related differences within the *Scn1b*-null population across any metric of myenteric neuron density, size, or excitatory/inhibitory ratios. These

data suggest that the observed dysmotility in P15+ *Scn1b*-null animals is not caused by aberrant myenteric neuron migration, size, or subtype expression in distal colon.

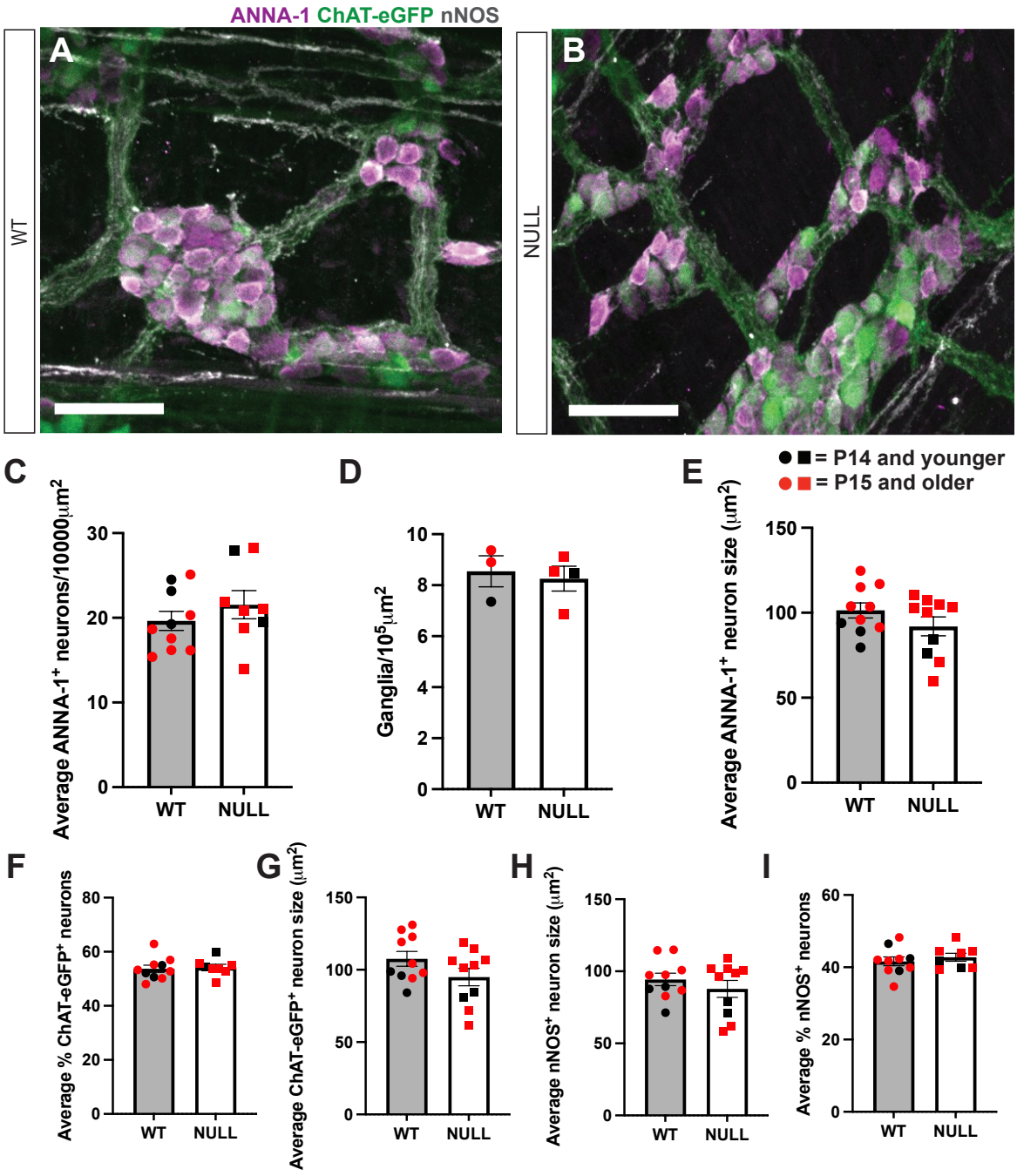


Figure 3.4. Scn1b-null mice have normal quantities, size, and proportions of myenteric neuron subtypes and ganglia. *A, B) Representative images taken of myenteric plexus in P14-P16 Scn1b-null and WT distal colons revealed C, D) no age- or genotype-dependent differences in myenteric neuron density, myenteric ganglion density, or average myenteric neuron size. Subsequent analysis of the two major subpopulations of myenteric neurons revealed no difference in size or ratio of F, G) excitatory (ChAT$^+$) myenteric neurons or H, I) inhibitory (nNOS$^+$) myenteric neurons. Scale bars: 50 μm. Data are represented as the mean ± SEM.*

Scn1b-null mice have altered myenteric plexus connectivity

Previous studies have shown that β1 subunits promote neurite outgrowth in CNS neurons (Davis et al., 2004; McEwen & Isom, 2004; Patino et al., 2011). These small immature extensions establish polarity, develop into axons and dendrites, and begin to form mature neural circuits. In the brain and spinal cord, β1 subunits also mediate axon fasciculation, the process by which growing axons adhere to one another to limit the dispersion of axonal tracks and generate more precise connections (Brackenbury et al., 2013). Proper excitatory and inhibitory connections within the myenteric plexus are necessary for highly stereotyped and coordinated motility patterns, and small changes in the organization of the plexus can produce large alterations in GI function (Sasselli et al., 2013). Therefore, we next sought to determine if *Scn1b*-null mice have normal axonal connections between myenteric ganglia in colon. Using anti-TUJ1 immunostaining to visualize axonal connections within the myenteric plexus of P14-P16 *Scn1b*-null and WT animals, we observed no difference in the average density of axonal connections between myenteric ganglia as well as no difference in the average length or width of the axonal bundles in *Scn1b*-null mice compared to WTs across all tested ages (Figure 3.5C-E). Each axon bundle in *Scn1b*-null and WT animals consisted of both excitatory (ChAT-eGFP$^+$) and inhibitory (nNOS$^+$) axons (Figures 3.5F, 3.5G). These data suggest loss of β1 subunits does not impact axonal network development within the ENS.

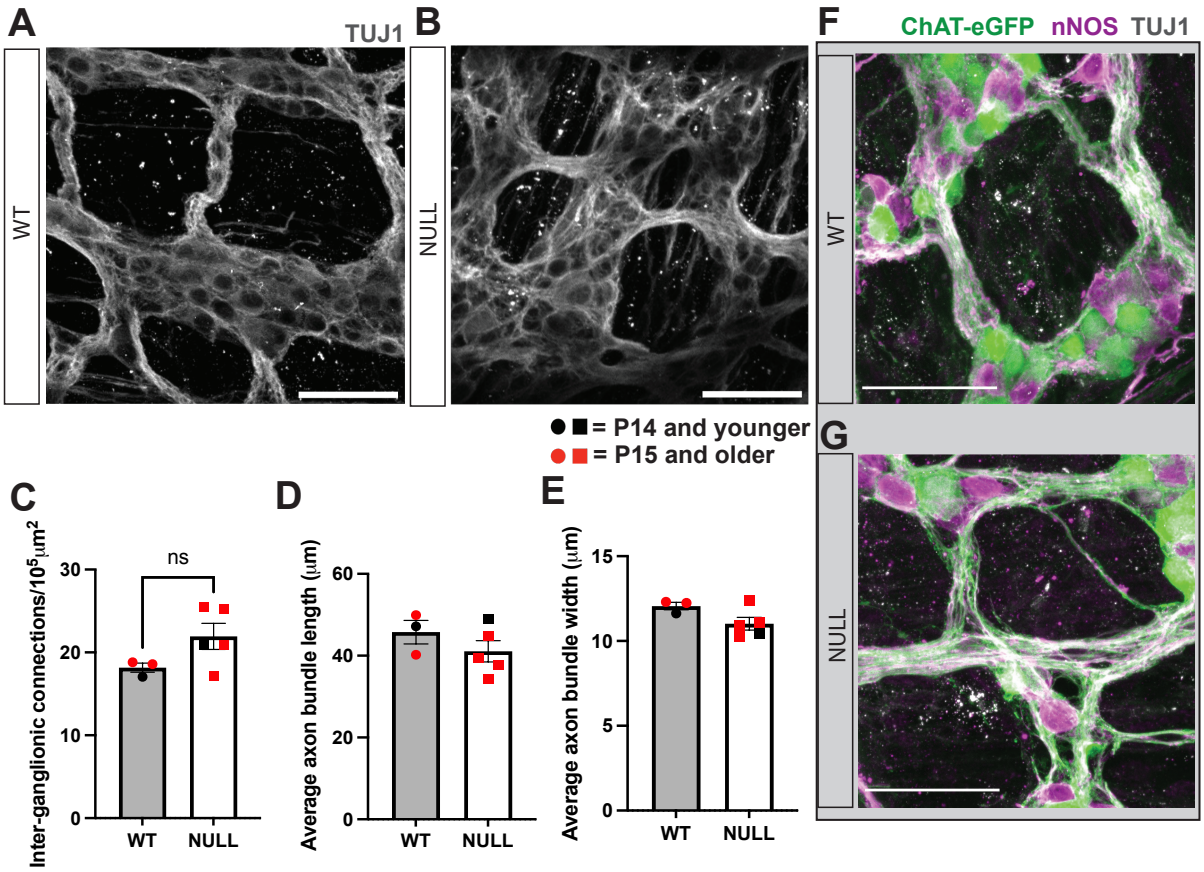


Figure 3.5: Scn1b-null mice exhibit normal inter-ganglionic axonal connections within the myenteric plexus. *A, B) Representative images taken of myenteric plexus connections in P14-P16 Scn1b-null and WT distal colons revealed no difference in C) average density of axonal bundles connecting myenteric ganglia as well as D) no difference in average bundle length or E) width. F, G) Representative images showing axon bundles in P14-P16 Scn1b-null and WT distal colon contain both ChAT$^+$ and nNOS$^+$ fibers. Scale bars: 50 μm. Data are represented as the mean ± SEM.*

Scn1b-null mice exhibit normal SMC responses to myenteric neuronal signaling

Our data suggest that the distal colons of P15+ *Scn1b*-null animals have dysregulated distension-induced peristatic contractions as well as aberrant expression of genes encoding VGICs necessary for myenteric neuron excitability and neurotransmitter release compared to WT animals. However, there were no evident differences in myenteric neuronal density or subpopulation ratios in distal colon between the two genotypes. Furthermore, *Scn1b*-null animals exhibited intact, properly sized, though slightly increased, axonal connections between myenteric ganglia. These results suggest that the mechanism of colonic dysmotility in *Scn1b*-null animals may include aberrant stimulus-induced inhibitory and/or excitatory signaling between myenteric neurons and SMCs. In mammals, proper inhibitory and excitatory myenteric neuron signaling to circular SMCs, but not longitudinal SMCs, in distal colon is necessary for distension-induced peristalsis (Spencer, Dickson, Hennig, & Smith, 2006). To test the integrity of signaling from myenteric neurons to circular SMCs in P15+ *Scn1b*-null animals, we performed sharp-electrode electrophysiological recordings in the distal colons of P15+ *Scn1b*-null and P14-P16 WT animals. We dissected distal colon sections containing longitudinal muscle, circular muscle, and myenteric plexus from these animals, and the circular SMCs were impaled with an intracellular microelectrode to record membrane potential. We then stimulated myenteric neurons in the tissue preparation using transmural electric field stimulation (EFS). Previous pharmacological studies show this stimulation intensity is below the threshold required to directly stimulate SMCs, so any observed changes in SMC membrane depolarization (inhibitory junction potential) or hyperpolarization (excitatory junction potential) following the stimulation was due to neuronal signaling to

SMCs (Perez-Medina & Galligan, 2019). Under baseline conditions, we observed no difference between the *Scn1b*-null and WT resting membrane potential (Figure 3.6B). Furthermore, we observed no difference in inhibitory junction potential size between P15+ *Scn1b*-null and P14-P16 WT animals in response to EFS (Figures 3.6Ai/ii, C, D). Likewise, we observed no difference in resting membrane potential nor in the relative reduction in inhibitory junction potential size under TTX conditions (Figures 3.6Ei/ii, F-H). Finally, we applied MRS 2179 and Nitro-L-Arginine (NLA) to block inhibitory junction potentials and unmask the excitatory junction potential response to EFS. Again, we observed no difference in resting membrane potential of *Scn1b*-null and WT SMCs under these conditions (Figure 3.6J). As with inhibitory signaling, we observed no difference in myenteric neuron excitatory signaling to SMCs between genotypes (Figures 3. 6Gi/ii, H, I). These data suggest that both inhibitory and excitatory myenteric neuron populations are able to signal properly to SMCs in response to electrical stimulation in P15+ *Scn1b*-null animals.

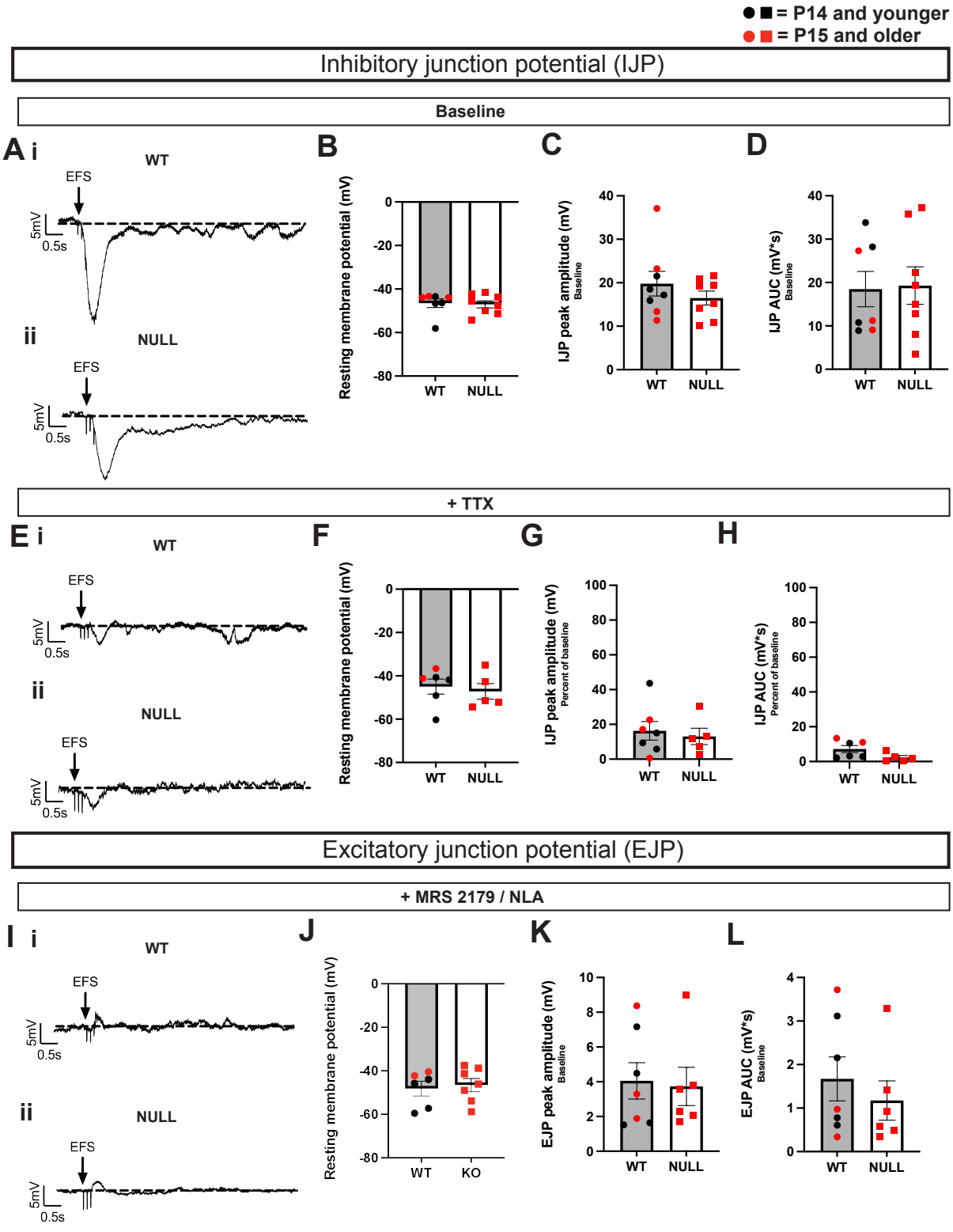


Figure 3.6: Scn1b-null mice exhibit no deficits in electrical stimulation-induced inhibitory or excitatory myenteric neuronal signaling to smooth muscle cells. *Electrophysiological recordings from smooth muscle cells in P15+ Scn1b-null and P14-*

P16 WT distal colon reveal reduction in A,C,D) inhibitory or I, K, L) excitatory signaling from myenteric neurons in response to electrical field stimulation (EFS). E,G,H) Additionally, there was no difference between genotypes in myenteric response to EFS in the presence of 300 nM TTX. Finally, there was no difference in SMC resting membrane potential between genotypes in any recording condition (B,F,J) Data are represented as the mean ± SEM.

3.5 Discussion

This project investigated a potential mechanism, aberrant activity of VGSC β1/β1B, that may underlie GI dysmotility symptoms commonly experienced by DEE patients with variants in *SCN1B* (DEE52). Based on the established roles of VGSC β1/β1B subunits in CNS activity and development, we also sought to determine if β1/β1B subunits played analogous parts in the ENS. Our data suggest that P15+ *Scn1b*-null mice mirror the constipation phenotype observed in DEE52 and other DEE channelopathy patients – and that this colonic dysmotility is driven by reduced and dysregulated peristaltic contractions within distal colon. Due to the absence of genotype-specific differences in the presence of TTX, we postulate that changes in neuronal excitability are responsible for the observed contractile dysfunction. Furthermore, our data suggest that differential mRNA abundance of VGSC and VGCC genes, which are necessary for proper enteric neuron excitability and signaling to effector SMCs, might contribute to the mechanism of dysmotility in *Scn1b*-null colon.

The observed colonic dysmotility phenotype appears to be developmentally regulated in the *Scn1b*-null model of DEE52: P14 *Scn1b*-null mice exhibit neither the colonic dysmotility patterns nor the altered gene transcription profiles found in P15+ *Scn1b*-null mice. As *Scn1b*-null animals age, they develop CNS hyperexcitability and FTT (Chen et al., 2004; Yuan et al., 2019). *Scn1b*-null animals exhibit more severe dysfunction that correlates with their disease onset by ~P12, disease progression, and eventual death by ~P19, thus it follows logically that ENS activity may similarly become increasingly irregular with age.

We observed aberrant expression of the VGIC-encoding genes *Scn9a*, *Scn11a*, *Cacna1b*, and *Cacna1e* in the distal colon of *Scn1b*-null mice. Many of these genes – as well as their respective protein products – are also found to be differentially expressed in *Scn1b*-null CNS, heart, or other subsets of PNS (Bouza et al., 2021; Lopez-Santiago et al., 2011). While the proteins encoded by *Scn11a*, *Cacna1b*, and *Cacna1e* have established roles in myenteric neuron signaling and GI smooth muscle motility (Bian et al., 2004; Copel et al., 2013; Osorio et al., 2014; Rugiero et al., 2002; Shuttleworth & Smith, 1999; Smith et al., 2003; Vogalis et al., 2002; Wessler et al., 1990), the protein product of *Scn9a* ($Na_v1.7$) does not yet have a known function in the GI system – though other researchers have reported *Scn9a* gene or $Na_v1.7$ protein expression in the GI tract of laboratory animals (Bartoo et al., 2005; Sage et al., 2007) and humans (Hetz et al., 2014). Unlike $Na_v1.5$ and $Na_v1.9$ (encoded by *Scn5a* and *Scn11a*, respectively), $Na_v1.7$ is a TTX-sensitive VGSC. Based on the effects of nanomolar TTX on enteric neuron action potential generation as well as patterns of colonic motility, there is substantial evidence for TTX-sensitive VGSC α subunit involvement in proper GI motility; however, the molecular identities of the TTX-sensitive VGSC(s) contributing to these activities are unknown (Fida et al., 1997; G. D. Hirst et al., 1974; Nakagawa et al., 2005). The significant upregulation of *Scn9a* in P15+ *Scn1b*-null mice may indicate the involvement of $Na_v1.7$ in regulated colonic contraction and relaxation patterns. Alternatively, this genetic upregulation could be compensatory to counter reductions in other VGIC activity in the *Scn1b*-null colon. Evidence of the latter scenario has previously been proposed in mice lacking *Cacna1a* (encoding the P/Q-type VGCC $Ca_v2.1$), which exhibit an enhancement

in the function of L-type VGCCs to maintain normal myenteric neuron inhibitory signaling (E. Rodriguez-Tapia et al., 2016).

We observed normal inhibitory and excitatory responses to electrical field stimulation in *Scn1b*-null SMCs. Though myenteric neuron signaling to circular smooth muscle is reportedly necessary and sufficient for distension-induced motility patterns (Spencer et al., 2006), it is possible that electrical stimulation bypasses dysfunctional mechanosensitive neuronal populations or induces firing in normally hypoexcitable myenteric neurons in the *Scn1b*-null model. Likewise, our baseline contraction amplitude data (Figure 3.2 C) taken with the measures of contraction regularity (Figures 3.2 E-G) show that – while stimulus-induced myenteric neuronal signaling to SMCs is just as robust in *Scn1b*-null mice as in WT mice when it occurs – this signaling is less frequent and less regular in *Scn1b*-null than in WTs. Both TTX-R and TTX-S VGSCs are also expressed in SMCs (Alberti et al., 2007; Berra-Romani, Blaustein, & Matteson, 2005; Beyder & Farrugia, 2012; Hetz et al., 2014; Holm et al., 2002; Neshatian et al., 2015; O'Donnell et al., 2016; Ou et al., 2002; Strege et al., 2007); however, we observe normal myogenic distension-induced contractions as well as resting membrane potential in SMCs in both baseline and TTX conditions, suggesting that intrinsic colonic smooth muscle excitability and contractility is unaffected in *Scn1b*-null animals. Due to the complex interplay of smooth muscle contractions and relaxations that produce highly stereotyped patterns of colonic motility, it is difficult to determine whether this observed dysmotility is due to aberrant excitatory signaling, inhibitory signaling, or both. A reduction in defined contractions could be due to a relative decrease in excitatory signaling events, an

unbalanced increase in basal inhibitory or excitatory tone, or even a reduction in inhibitory signaling events, resulting in depolarization block of SMCs such that they cannot produce contractions in response to excitatory neurotransmission. To more directly address the mechanisms underlying the observed colonic dysmotility in *Scn1b*-null mice, future work will need to examine individual excitatory and inhibitory myenteric neuron excitability in *Scn1b*-null and WT distal colon via whole-cell patch clamp experiments.

In conclusion, this work supports the idea that GI symptoms in channelopathies such as DEE may be caused by expression of the particular ion channel gene variant in ENS neurons, resulting in aberrant motility and functionality of the GI tract. Furthermore, our data suggest that *SCN1B* has analogous roles in the CNS and ENS – impacting neuronal excitability, VGIC gene expression, and network connectivity.

Chapter 4 : Future Directions

4.1 Introduction

While the primary focus of this thesis has been to understand the role of *Scn1b* in myenteric neurons, pacemaker cells, and SMCs of the distal colon, it is important to remember that the GI system contains myriad cell types – many of which are also known to express VGSCs. It is therefore logical that aberrant VGSC expression or function due to *SCN1B* disease variants could impact other functions vital to normal GI function. In this final chapter, I will elaborate on some potential additional roles of VGSC β1/β1B subunits in the GI system – including preliminary data from our laboratory – as well as some potential avenues of future GI research in our mouse model of DEE52:

4.2: DEE52 patients and *Scn1b*-null mice exhibit severe failure-to-thrive. VGSC β1 subunits may play a role in proper development, nutrient absorption, and/or motility of the small intestine.

4.3: The vagus nerve serves as one of the primary routes of communication between the CNS and the GI system. In mice, VGSC β1 subunits are known to be expressed at AIS and nodes of Ranvier in the vagus nerve, which enable the conduction of nerve signals over long distances. VGSC β1 subunits may play a role in the proper functioning of the vagal connection between brain and gut.

4.4: The gut microbiome is necessary for proper GI – and potentially CNS – function, and its composition can be disrupted in genetic disorders and various epilepsies. The gut microbiome may be altered in DEE52 patients and *Scn1b*-null mice, contributing to malabsorption and dysmotility.

4.5: The ketogenic diet is known to be therapeutic in many forms of epilepsy, including DEEs; however, the impact of the diet on longevity and other relevant metrics in *Scn1b*-null mice has yet to be investigated.

4.2 Elucidating the Role of *Scn1b* in Nutrient Absorption and Metabolic Function

As discussed in Chapter 1, most of the body's absorption of macronutrients (carbohydrates, proteins, and lipids) and micronutrients (vitamins and minerals) during digestion occurs in the small intestine, and this process involves the submucosal plexus (Fish & Burns, 2021; Ogobuiro et al., 2021). Luminal stimuli, such as mechanical distension or the acid and nutrients in digested food that enters the duodenum from the stomach, prompt enterochromaffin cells to release 5-HT and ATP to submucosal afferent neurons (Xue, Askwith, Javed, & Cooke, 2007). Activated secretomotor neurons in the submucosal plexus release SP, VIP, and ACh - which bind to receptors on epithelial cells and stimulate secretions into the intestinal lumen (Furness, 2007; Hansen, 2003; Hansen & Skadhauge, 1997; Reed & Vanner, 2001, 2003; Xue et al., 2007). Enterochromaffin cells and submucosal neurons express VGSCs (Bartoo et al., 2005; Padilla et al., 2007; Strege et al., 2017). Therefore, aberrant VGSC expression or function due to *SCN1B* disease variants could impair nutrient absorption and extraction by impacting the excitability of enterochromaffin cells and/or submucosal enteric neurons.

DEE52 patients, as well as patients with DEEs linked to other VGSC gene variants, exhibit a striking reduction in developmental growth and weight gain, ie. failure-to-thrive (FTT), despite clinical reports of adequate caloric intake (Personal_Communication, 2019 & 2020) (Table 1.1). *Scn1b*-null mice also exhibit a FTT phenotype, despite the aforementioned observations of constant feeding (Figure 3.1 A, Figure 4.1 A, B) (Chen et al., 2004; Yuan et al., 2019).

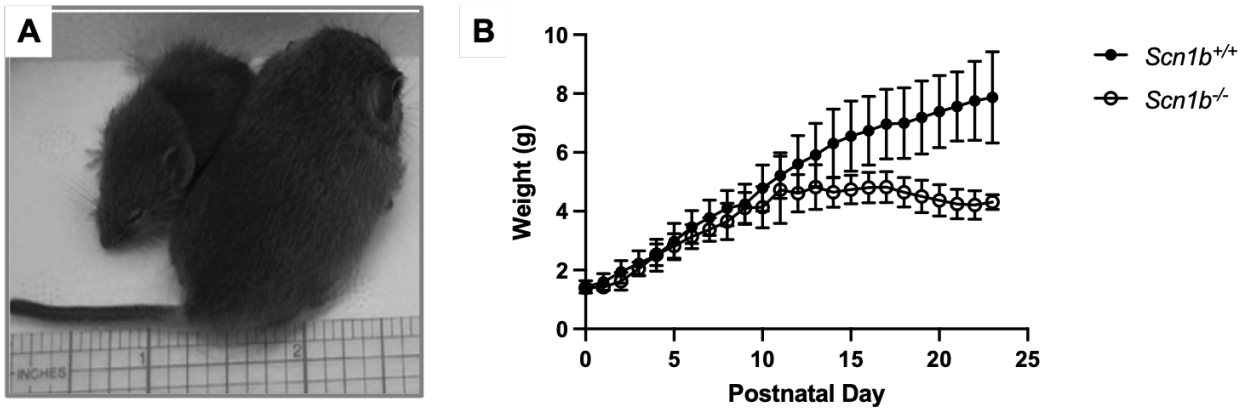


Figure 4.1:* Scn1b-*null mice exhibit a failure-to-thrive phenotype. *A) P19* Scn1b-*null mouse compared to a WT littermate. B)* Scn1b-*null mice exhibit a marked decrease in developmental weight gain, with weights beginning to deviate from age-matched WT mice around P12. Picture initially published in Chen et al. (2002); weight data collected during study published in Yuan, O'Malley et al. (2019).*

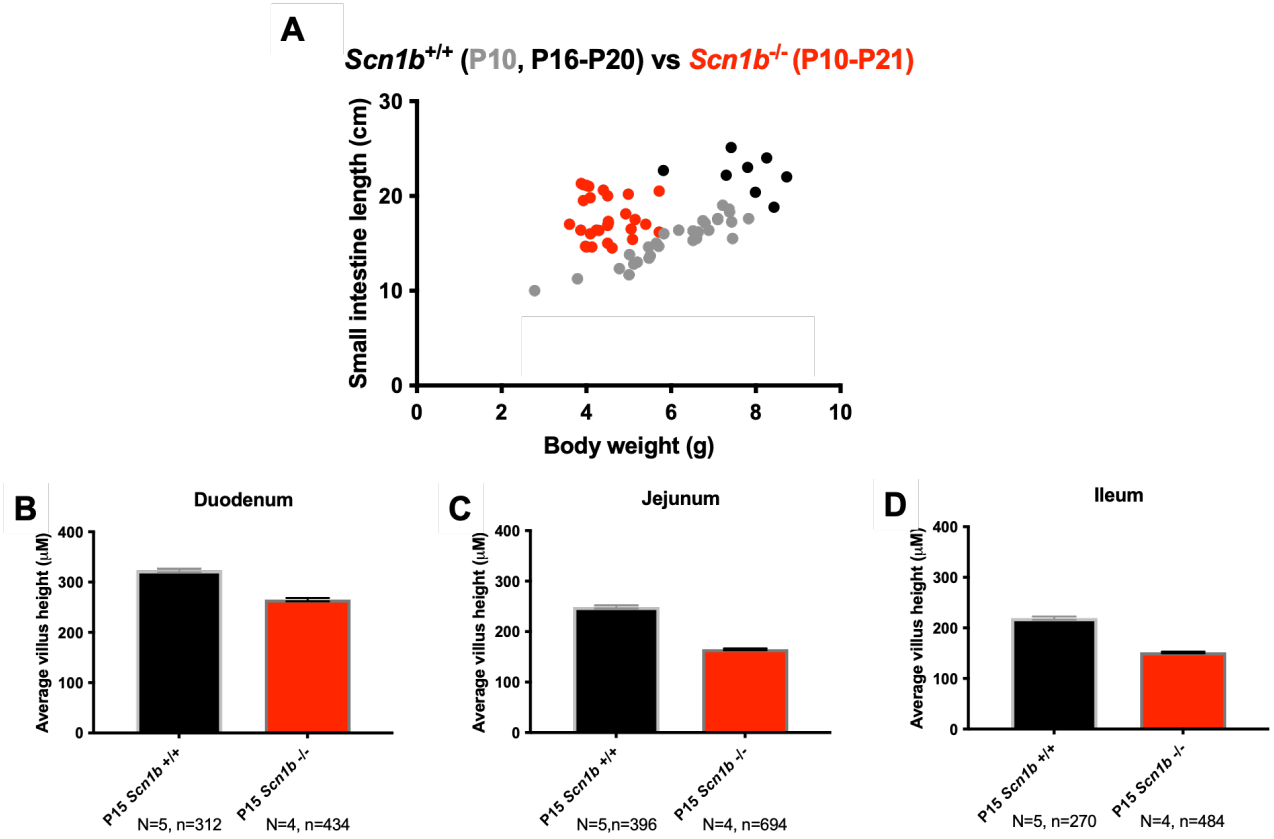


Figure 4.2:* Scn1b-*null animals exhibit aberrant GI tract morphology. *Loss of* Scn1b *results in (A) a higher small-intestine length to body weight ratio and B) a slight reduction in average villus height in all three sections of the small intestine.*

Aside from the evident FTT observed in *Scn1b*-null mice, preliminary morphological evaluations of the *Scn1b*-null small intestine suggest potential aberrant nutrient absorption capabilities. I observed a relative increase in *Scn1b*-null small intestine length to body weight ratio compared to WT littermates of either the same age or to WTs of similar body weight (Figure 4.2 A). This suggests that *Scn1b*-null animals continue to devote energy resources to elongating their GI tract, even after their overall developmental weight gain has stagnated around P11 or P12. This relative increase in energy allocation may serve as a compensatory mechanism to offset FTT, as a longer small intestine would allow for more surface area available for nutrient absorption. Further

morphological analysis of the small intestine found a reduction in average villus height in all three sections (Figure 4.2 B-D), and preliminary data from *Scn1b*-null mice, performed in conjunction with the Adipose Tissue Core within the Michigan Nutrition Obesity Center, also suggest defects in lipid absorption (Figure 4.3). These findings suggest that *Scn1b*-null animals may exhibit FTT due to defects in macronutrient absorption through their intestinal villi.

Results of the Oil Red O staining experiment (results shown in Figure 4.3) could be verified in future work by performing the same analysis on fecal smear samples from *Scn1b*-null and WT mice. If fecal smears from null mice show a larger lipid accumulation, it would further support the idea that *Scn1b*-null mice have aberrant intestinal lipid absorption rather than deficits in dietary lipid intake. Future experiments could also investigate relative caloric absorption within the GI tract of *Scn1b*-null and WT mice by performing bomb calorimetry on fecal samples in partnership with the University of Michigan Nutrition Obesity Center's Animal Phenotyping Core. These latter experiments would enable us to understand general macronutrient absorption in the intestines of *Scn1b*-null mice versus their WT littermates.

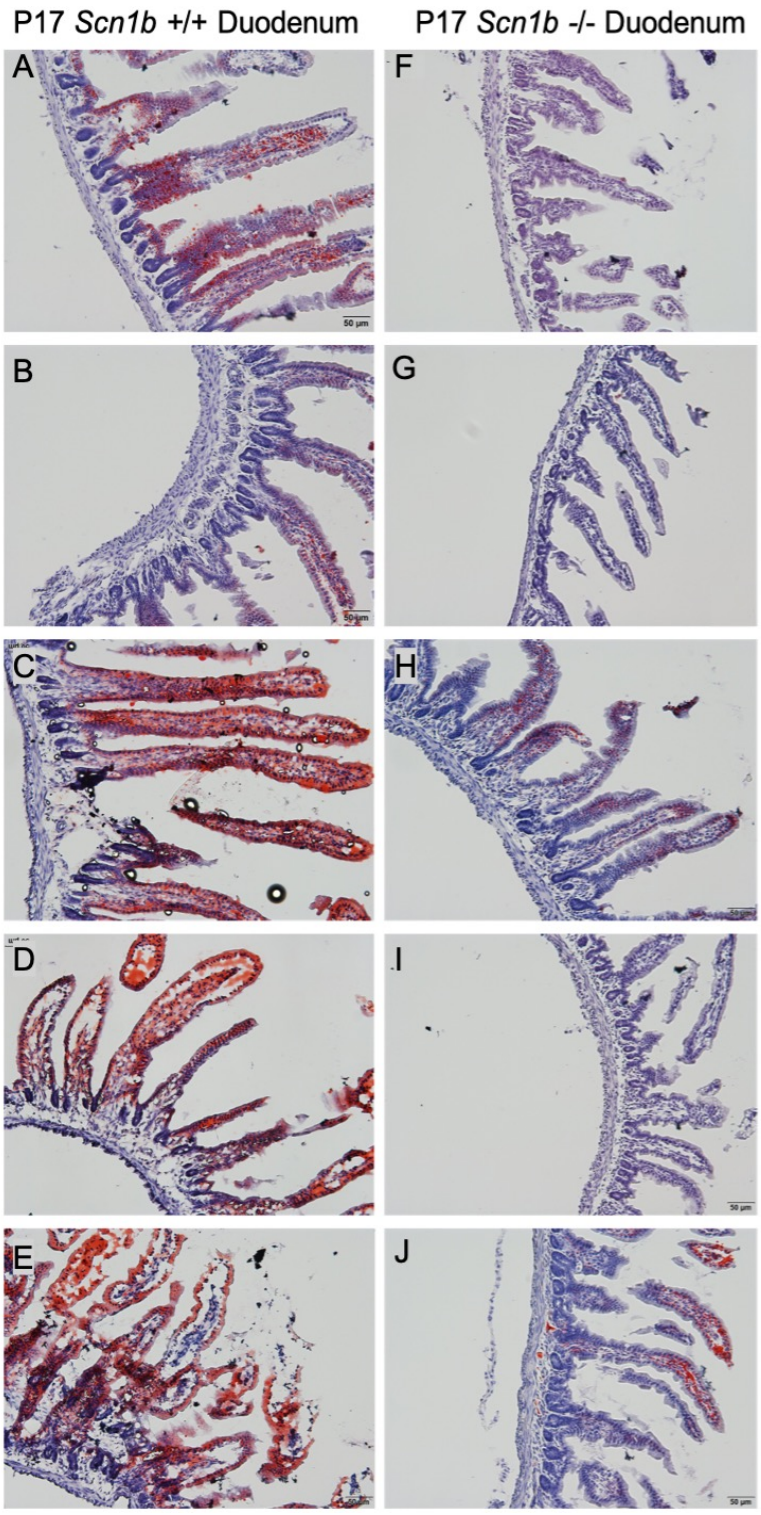


Figure 4.3:* Scn1b-*null mice exhibit evidence of macronutrient malabsorption.
Scn1b-*null mice (F-J) exhibit decreased intestinal lipid accumulation (shown by Oil Red O staining) compared to WT mice (A-E) in duodenal sections of small intestine.*

We performed metabolic analyses in conjunction with the University of Michigan Small Animal Phenotyping Core, including NMR spectroscopy to provide information on the chemical structure of *Scn1b*-null and age-matched WT tissues (Figure 4.4 A-C). These experiments were performed at two ages: P10, which is prior to the onset of the FTT phenotype in *Scn1b*-null mice, and P15, at which point the weights of *Scn1b*-null mice and their WT littermates have begun to deviate significantly. These data suggest that *Scn1b*-null mice exhibit an age-dependent decrease in proportion of lean tissue, such as skeletal muscle, which could result from the improper absorption or cellular uptake of macronutrients such as proteins. We were surprised to see that, at both ages, *Scn1b*-null mice exhibited a larger percentage of fat tissue mass compared to their overall body mass. Anecdotal evidence from numerous dissections from these animals suggested a visible reduction in white adipose tissue surrounding the internal organs of *Scn1b*-null mice. To begin to address this apparent discrepancy, further research could probe potential differences in location of fat accumulation as well as precise differences in fatty tissue composition/metabolic activity between *Scn1b*-null and WT animals.

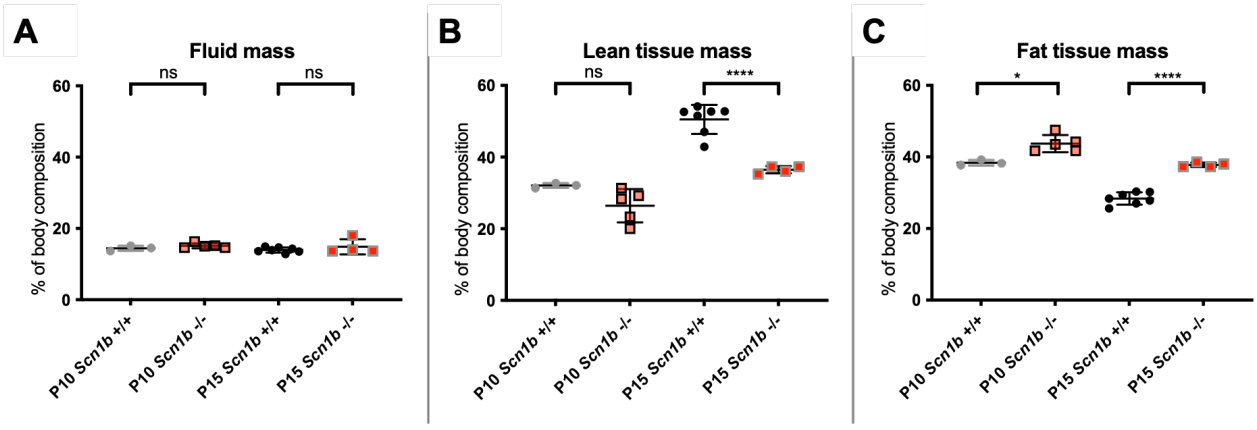


Figure 4.4: Scn1b-null mice exhibit aberrant body tissue composition, as measured by NMR. *Scn1b-null mice exhibit age-dependent and genotype-dependent changes in relative lean and fat tissue mass*.

Finally, it is possible that *Scn1b* plays a similar role within the myenteric neuron population in the small intestine as it does in the large intestine – facilitating the intricate motility patterns that are necessary for proper digestion. Migrating motor complexes and peristalsis also occur in the small intestine, and organ bath experiments – similar to those discussed in Chapter 3 – could compare the contraction patterns of duodenum, jejunum, and ileum segments from *Scn1b*-null and WT animals. Furthermore, we could measure GI motility by comparing the rate of gastric emptying in age-matched *Scn1b*-null and WT mice. Mice would be administered an oral gavage of a non-nutrient dye, and intestinal transit would be assessed at a set time point after gavage. Aberrant small intestine motility patterns and transit times could produce the FTT phenotype; increased rates of intestinal motility could shunt digested material through the small intestine too quickly to be effectively absorbed, while decreased rates of intestinal motility could lead to bacterial overgrowth (SIBO) – leading to malabsorption (Dukowicz, Lacy, & Levine, 2007; Keller & Layer, 2014).

4.3 Investigating the Function of *Scn1b* in Gut-Brain Communication

While the ENS can control many GI functions in isolation, the digestive tract relies on dual innervation from the ENS and CNS, as well as communication between these two nervous systems, for normal physiological functioning (Gershon, 1999). The most direct route of contact between the GI tract and the CNS is the vagus nerve. Vagal efferent fibers, originating from neurons in the dorsal motor nucleus of the vagus (DMV), help to regulate esophageal propulsion, gastric emptying, contractile activity (via complexes with myenteric motor neurons, ICCs, and SMCs), and acid secretion (Masuda, Tomita, Okubo, & Miyasaka, 1994; Patino & Isom, 2010; Powley & Phillips, 2011). Vagal afferent fibers relay information to the CNS regarding GI tract distension as well as the presence of hormones and macronutrients within the tract, leading to regulation of hunger and satiety sensations (Furness et al., 2014; Masuda et al., 1994; Powley & Phillips, 2011).

Scn1b-null mice have significantly fewer nodes of Ranvier in the optic nerve with functional deficits in optic nerve conduction (Chen et al., 2004). Preliminary work shows a similar significant reduction in nodes of Ranvier in *Scn1b*-null corpus collosum compared to WT ($P = 0.0357$; Figure 4.5). The vagus nerve expresses VGSC α and β1 subunits at nodes of Ranvier (Figure 4.6), thus loss of β1 subunits may impair conduction of neural signals along the vagus nerve, reducing vagal input to enteric neurons and pacemaker cells and impairing proper digestion.

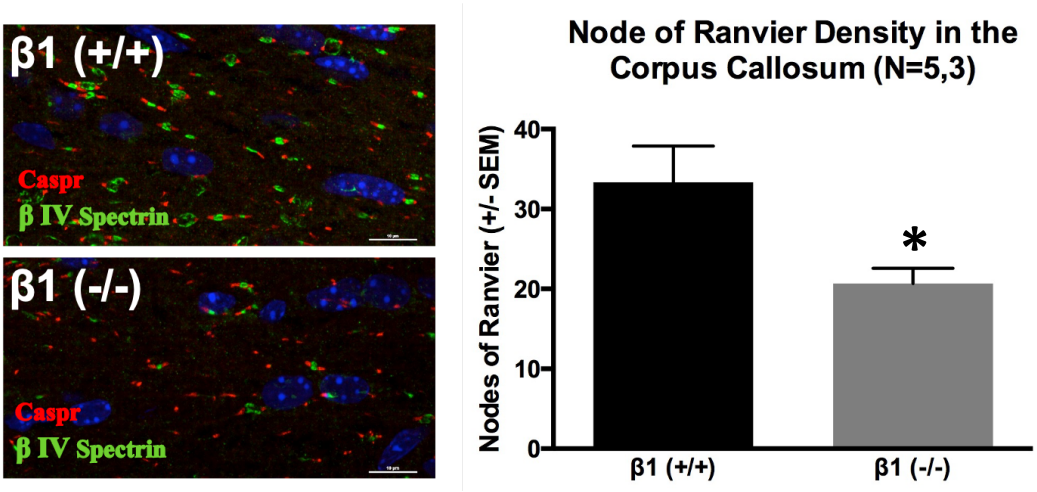


Figure 4.5:* P17 *Scn1b-null mice exhibit a significant reduction (P = 0.0357) in nodes of Ranvier density in the corpus callosum compared to age-matched WTs. *Scale bars = 10μm. * = P < 0.05 .Data courtesy of A. De La Rocha.*

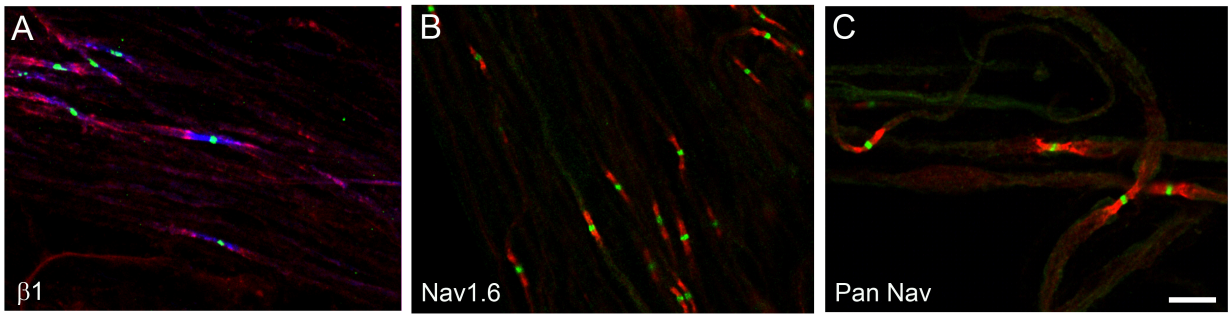


***Figure 4.6: Mouse vagus nerve expresses VGSC β and α subunits**. A) β1 subunits (green) at nodes of Ranvier (anti-Caspr antibody, blue) and flanked by K_v 1.2 channels (red). B,C) α subunits (green) at nodes of Ranvier (anti-Caspr, red). Scale bar = 10μm. Data courtesy of Dr. H.A. O'Malley.*

The vagus nerve innervates multiple GI targets, suggesting there are precise mechanisms that guide vagal axons to establish ENS-CNS innervation patterns. As previously discussed, β1/β1B subunits are CAMs that regulate cell adhesion and neuronal migration/axonal pathfinding (Brackenbury & Isom, 2011). Previous studies have revealed that both β1 and its soluble splice variant, β1B, promote neurite outgrowth in cerebellar granule neurons via CAM-mediated signaling through the lipid raft-

associated non-receptor tyrosine kinase fyn signaling pathway following *trans* homophilic cell-cell adhesion (Davis et al., 2004; McEwen & Isom, 2004). The small neurite extensions that protrude from immature neurons establish polarity, develop into axons and dendrites, and begin to form the connections that eventually define neural circuits. In the CNS, *Scn1b* mediates axon fasciculation, the process by which growing axons adhere to one another to limit the dispersion of axonal tracks and generate more precise connections between regions. *Scn1b*-null mice, which lack β1/β1B-mediated neuronal outgrowth, exhibit disrupted axonal pathfinding in the cerebellum as well as in the corticospinal tract (Brackenbury et al., 2008). Therefore, we predict that *Scn1b*-null mice might also have impaired pathfinding of vagal nerve fibers.

Previous research has suggested that brain-derived neurotrophic factor (BDNF) plays a critical role in vagal innervation. BDNF, which is expressed during gut development in tissues adjacent to sensory and motor vagal innervation, has been shown to play a role in vagal axon growth, guidance, or fasciculation — as mice lacking BDNF display aberrant patterning of axon bundles in the gastric antrum (Murphy & Fox, 2010). Fyn kinase, which is necessary for β1-mediated neurite outgrowth in the CNS, is responsible for regulating BDNF receptor (TrkB) localization in lipid rafts and subsequent signaling in neurons (Pereira & Chao, 2007). Fyn kinase is also known to modulate the activity of VGSCs in response to BDNF signaling (Ahn, Beacham, Westenbroek, Scheuer, & Catterall, 2007). Loss of β1/β1B subunit function could therefore impair the formation of these complex innervation patterns, which are necessary for normal functioning of the CNS, ENS, and GI tract. However, whether β1/β1B subunits also mediate axonal tract development and

conduction in the nerve tracts between CNS and ENS, and therefore the extent to which *Scn1b*-null disease variants may affect the normal vagal innervation of and transmission to the GI tract, has yet to be investigated.

Future studies could therefore examine the effect of *Scn1b* deletion on vagal development and conduction. By dissecting vagus nerves from age-matched *Scn1b*-null and WT mice and then utilizing immunohistochemistry and confocal imaging to quantify changes in axonal organization, localization and expression levels of different VGSC α subunits, potassium channels, and CAMs, as well as numbers of nodes of Ranvier, we could characterize differences in vagus nerve development and fasciculation between genotypes. Future work could also assess genotypic differences I_{Na} density and conduction velocity in the vagus nerve. Another line of investigation could seek to establish the effect of *Scn1b* deletion on the extent and patterning of vagal innervation of the stomach and GI tract. Using *Scn1b*-null and WT mice expressing the fluorescent protein tdTomato driven by the *Phox2b* promoter, which is expressed by mammalian DMV neurons (Kang et al., 2007), points of DMV innervation on stomach and intestine could potentially be quantified via confocal microscopy (Udit & Gautron, 2013).

4.4 Establishing the Gut Microbiome Profile of *Scn1b*-null Mice

The gut microbiome is involved in several vital functions, including maintenance of GI tract motility as well as the synthesis and metabolism of nutrients, vitamins, and drugs (Dieterich, Schink, & Zopf, 2018; Quigley, 2011; Rowland et al., 2018; Sun, Chen, & Shen, 2019). Though infants receive their initial microbiome from their mothers, compositions of gut microbiota change with age, and overall heritability of the gut microbiome is very low (Backhed et al., 2015; Stanislawski et al., 2017; Turnbaugh et al., 2009; H. X. Wang & Wang, 2016). Multiple factors have been found to impact the distribution and diversity of species in the gut microbiome, including infections, stress, antibiotics, and chronic constipation (Azad, Bridgman, Becker, & Kozyrskyj, 2014; Galley et al., 2014; Ohkusa, Koido, Nishikawa, & Sato, 2019). Certain genetic variants are also linked to microbiome alterations in humans, including variants that alter the function of genes encoding ion channels (Hall, Tolonen, & Xavier, 2017). Cystic fibrosis, a channelopathy caused by variants in the gene encoding the cystic fibrosis transmembrane conductance regulator, is associated with altered intestinal microbiota (Fiorotto & Strazzabosco, 2019). Nonetheless, diet is the most important determinant of gut microbiota composition (Azad et al., 2014; Hooper et al., 2001).

Recent studies have suggested that the connection between the gut microbiome and the brain might be relevant for neurological disease. Around 25% of autistic patients have at least one type of GI disease, and these patients have been reported to exhibit altered microbiome compositions by multiple studies (Morais, Schreiber, & Mazmanian, 2021).

There is also evidence of associations between aberrant microbiota compositions in patients with multiple sclerosis, Parkinson's disease, Alzheimer's disease, as well as anxiety, depression, and stress (Dieterich et al., 2018; Mazurek et al., 2013; Morais et al., 2021; X. Zhu et al., 2017).

Finally, there is some evidence supporting microbiome involvement in epilepsy. A few human studies have found differences in the fecal microbiota of refractory epilepsy patients compared to their healthy peers (Lindefeldt et al., 2019; Lum, Olson, & Hsiao, 2020; Peng et al., 2018; Xie et al., 2017). There is even a potential difference in the microbiome composition between patients with drug-resistant and drug-sensitive epilepsy (Dahlin & Prast-Nielsen, 2019; Peng et al., 2018). Fecal transplants, probiotic, and antibiotic treatments have been recorded to have anticonvulsant effects (Braakman & van Ingen, 2018; Dahlin & Prast-Nielsen, 2019; Gomez-Eguilaz, Ramon-Trapero, Perez-Martinez, & Blanco, 2018; He et al., 2017). Future studies could examine the fecal microbiome of *Scn1b*-null mice compared to their WT littermates before and after seizure onset and developmental weight gain deviation around P12. Furthermore, it would be enlightening to perform functional GI motility experiments on the mice (such as those described in Chapter 3) following fecal microbiome analysis to determine if any alterations in microbiota correlate to severity of colonic dysmotility.

4.5 Determining Effect of Ketogenic Diet Intervention on *Scn1b*-null Mice

Based on the knowledge that the microbiome both influences neuronal function and is involved in numerous CNS disorders, it is reasonable to infer that changes in diet may serve as therapies in the treatment of disease. Studies have revealed that regular consumption of certain macronutrients can affect the prevalence of specific bacterial phyla in the microbiome (Lindefeldt et al., 2019; G. D. Wu et al., 2011). A common alternative treatment for intractable epilepsy patients, including DEE patients, is the high-fat, low-carbohydrate ketogenic diet (KD). Fasting has been used as form of treatment for epilepsy since the time of Hippocrates in 5th century BC, and the KD was first formulated in 1921 to induce the metabolic effects of fasting for the management of seizures (Caraballo et al., 2005). Use of the KD induces ketosis, in which dietary fat is converted to ketone bodies (β-hydroxybutyrate, acetoacetate, and acetone) in the liver as an alternative energy source to carbohydrates (Dahlin & Prast-Nielsen, 2019). The resulting metabolic alterations are thought to produce neuroprotective effects. In multiple studies, this diet-based therapy has been found to both reduce the frequency of a wide range of seizure types as well as produce improvements in cognition and behavior in DEE patients (Caraballo et al., 2005; Sharma & Tripathi, 2013; Y. Q. Wang, Fang, Zhang, Xie, & Jiang, 2020). There is also a report of the KD being used successfully in a patient with DEE52 (Aeby et al., 2019).

It has been proposed that the therapeutic effects of the KD are due to increased inhibitory neurotransmitter (GABA) signaling and/or decreased excitatory neurotransmitter

(glutamate) signaling, in response to diet-induced changes in intestinal microbiota, metabolism, and subsequent neurotransmitter production (Dahlin, Elfving, Ungerstedt, & Amark, 2005; Olson et al., 2018; Spinelli & Blackford, 2018; Z. J. Wang et al., 2003; Yudkoff et al., 2005). A recent study showed that the ketone bodies β-hydroxybutyrate and acetoacetate suppress glutamate release by directly competing with a positive allosteric regulator of the vesicular glutamate transporters in neurons (Juge et al., 2010).

It has also been suggested that mitochondria play a vital role in the antiepileptic effects of ketone bodies (Rogawski, Loscher, & Rho, 2016). Mitochondria are ATP-producing organelles, and reduced levels of ATP production are associated with increased seizure susceptibility due to an unstable membrane potential that precedes aberrant neuronal firing patterns (Bough et al., 2006; Rogawski et al., 2016). Additionally, clinical studies reveal a comorbidity between mitochondrial diseases and epilepsy (Craig, de Menezes, & Saneto, 2012). Previous research showed that the KD is associated with a drastic upregulation of transcription for genes encoding metabolic enzymes and mitochondrial proteins, as well as an increase in mitochondrial biogenesis in the hippocampus—a known seizure trigger zone (Bough et al., 2006).

Future experiments could explore the use of this diet in our *Scn1b*-null mice and allow us to better understand the mechanism by which the KD exerts its therapeutic effects in DEE52 patients. Because our *Scn1b*-null mice typically die before P20 and thus do not survive past weaning, we could study the effect of the KD in our *Scn1b*-flox mouse line expressing a ubiquitous tamoxifen-inducible Cre (e.g. CMV or ubiquitin). We have shown

previously that *Scn1b*$^{flox/flox}$/CMV-Cre mice have a phenotype that is indistinguishable from *Scn1b*- null mice (Chen et al., 2004). *Scn1b*$^{flox/flox}$ mice expressing a Thy1-promoter driven, tamoxifen-inducible Cre develop severe seizures and SUDEP within 10 days of tamoxifen injection (O'Malley et al., 2019). Therefore, using an inducible line, *Scn1b* can be deleted after the mice are weaned to determine if the KD results in amelioration in seizure severity or a reduction in SUDEP occurrence. If an amelioratory effect of the KD is observed in our *Scn1b*-null animals, we can further probe potential diet-mediated mechanisms, such as alterations in gene transcription and functional shifts in the animals' fecal and gut microbiome profiles.

4.6 Final Conclusions

Increasing evidence suggests that there is a connection between GI and neurological dysfunction in many diseases, including DEEs. The data I have presented in this thesis suggest that aberrant function of VGSC α and β1 subunits may provide a mechanism for comorbid GI symptoms in DEE patients and other patients with VGSC-linked diseases. Specifically, my results support novel roles for *Scn1b* in the coordination of enteric neuron function and colonic motility. Future studies that seek to better understand the many roles of VGSC β1 subunits in both normal GI physiology as well as the aberrant GI physiology observed in VGSC-linked neurological and GI diseases can inform more effective treatments for these patients and improve quality of life for both patients and caregivers.

Bibliography

Aeby, A., Sculier, C., Bouza, A. A., Askar, B., Lederer, D., Schoonjans, A. S., . . . Isom, L. L. (2019). SCN1B-linked early infantile developmental and epileptic encephalopathy. *Ann Clin Transl Neurol, 6*(12), 2354-2367. doi:10.1002/acn3.50921

Ahn, M., Beacham, D., Westenbroek, R. E., Scheuer, T., & Catterall, W. A. (2007). Regulation of Na(v)1.2 channels by brain-derived neurotrophic factor, TrkB, and associated Fyn kinase. *J Neurosci, 27*(43), 11533-11542. doi:10.1523/JNEUROSCI.5005-06.2007

Al-Mehmadi, S., Splitt, M., For, D. D. D. S. g., Ramesh, V., DeBrosse, S., Dessoffy, K., . . . For, C. S. g. d. (2016). FHF1 (FGF12) epileptic encephalopathy. *Neurol Genet, 2*(6), e115. doi:10.1212/NXG.0000000000000115

Alberti, E., Mikkelsen, H. B., Wang, X. Y., Diaz, M., Larsen, J. O., Huizinga, J. D., & Jimenez, M. (2007). Pacemaker activity and inhibitory neurotransmission in the colon of Ws/Ws mutant rats. *Am J Physiol Gastrointest Liver Physiol, 292*(6), G1499-1510. doi:10.1152/ajpgi.00136.2006

Alcaino, C., Knutson, K. R., Treichel, A. J., Yildiz, G., Strege, P. R., Linden, D. R., . . . Beyder, A. (2018). A population of gut epithelial enterochromaffin cells is mechanosensitive and requires Piezo2 to convert force into serotonin release. *Proc Natl Acad Sci U S A, 115*(32), E7632-E7641. doi:10.1073/pnas.1804938115

Amberg, G. C., Koh, S. D., Imaizumi, Y., Ohya, S., & Sanders, K. M. (2003). A-type potassium currents in smooth muscle. *Am J Physiol Cell Physiol, 284*(3), C583-595. doi:10.1152/ajpcell.00301.2002

Aronica, E., Troost, D., Rozemuller, A. J., Yankaya, B., Jansen, G. H., Isom, L. L., & Gorter, J. A. (2003). Expression and regulation of voltage-gated sodium channel beta1 subunit protein in human gliosis-associated pathologies. *Acta Neuropathol, 105*(5), 515-523. doi:10.1007/s00401-003-0677-2

Avetisyan, M., Schill, E. M., & Heuckeroth, R. O. (2015). Building a second brain in the bowel. *J Clin Invest, 125*(3), 899-907. doi:10.1172/JCI76307

Azad, M. B., Bridgman, S. L., Becker, A. B., & Kozyrskyj, A. L. (2014). Infant antibiotic exposure and the development of childhood overweight and central adiposity. *Int J Obes (Lond), 38*(10), 1290-1298. doi:10.1038/ijo.2014.119

Backhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., . . . Wang, J. (2015). Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. *Cell Host Microbe, 17*(6), 852. doi:10.1016/j.chom.2015.05.012

Barshack, I., Levite, M., Lang, A., Fudim, E., Picard, O., Ben Horin, S., & Chowers, Y. (2008). Functional voltage-gated sodium channels are expressed in human intestinal epithelial cells. *Digestion, 77*(2), 108-117. doi:10.1159/000123840

Bartoo, A. C., Sprunger, L. K., & Schneider, D. A. (2005). Expression and distribution of TTX-sensitive sodium channel alpha subunits in the enteric nervous system. *J Comp Neurol, 486*(2), 117-131. doi:10.1002/cne.20541

Bassotti, G., De Giorgio, R., Stanghellini, V., Tonini, M., Barbara, G., Salvioli, B., . . . Corinaldesi, R. (1998). Constipation: a common problem in patients with neurological abnormalities. *Ital J Gastroenterol Hepatol, 30*(5), 542-548. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/9836115

Beck, V. C., Isom, L. L., & Berg, A. T. (2021). Gastrointestinal symptoms and channelopathy-associated epilepsy. *J Pediatr*. doi:10.1016/j.jpeds.2021.06.034

Beckett, E. A., Young, H. M., Bornstein, J. C., & Jadcherla, S. R. (2017). Development of Gut Motility. In T. N. Faure C., Di Lorenzo C. (Ed.), *Pediatric Neurogastroenterology*: Springer, Cham.

Berecki, G., Howell, K. B., Deerasooriya, Y. H., Cilio, M. R., Oliva, M. K., Kaplan, D., . . . Petrou, S. (2018). Dynamic action potential clamp predicts functional separation in mild familial and severe de novo forms of SCN2A epilepsy. *Proc Natl Acad Sci U S A, 115*(24), E5516-E5525. doi:10.1073/pnas.1800077115

Berg, A. T., Gaebler, D., Wilkening, G., Zelko, F., Knupp, K., Dixon-Salazar, T., . . . Nesbitt, G. (2020). Nonseizure consequences of Dravet syndrome, *KCNQ2*-DEE, *KCNB1*-DEE, Lennox-Gastaut syndrome, ESES: A functional framework. *Epilepsy & Behavior, Online ahead of print*. doi:10.1016/j.yebeh.2020.107287

Berg, A. T., Tarquinio, D., & Koh, S. (2017). Early Life Epilepsies are a Comorbidity of Developmental Brain Disorders. *Semin Pediatr Neurol, 24*(4), 251-263. doi:10.1016/j.spen.2017.10.008

Berra-Romani, R., Blaustein, M. P., & Matteson, D. R. (2005). TTX-sensitive voltage-gated Na+ channels are expressed in mesenteric artery smooth muscle cells. *Am J Physiol Heart Circ Physiol, 289*(1), H137-145. doi:10.1152/ajpheart.01156.2004

Bertrand, P. P. (2004). Real-time detection of serotonin release from enterochromaffin cells of the guinea-pig ileum. *Neurogastroenterol Motil, 16*(5), 511-514. doi:10.1111/j.1365-2982.2004.00572.x

Beyder, A., & Farrugia, G. (2012). Targeting ion channels for the treatment of gastrointestinal motility disorders. *Therap Adv Gastroenterol, 5*(1), 5-21. doi:10.1177/1756283X11415892

Beyder, A., & Farrugia, G. (2016). Ion channelopathies in functional GI disorders. *Am J Physiol Gastrointest Liver Physiol, 311*(4), G581-G586. doi:10.1152/ajpgi.00237.2016

Beyder, A., Mazzone, A., Strege, P. R., Tester, D. J., Saito, Y. A., Bernard, C. E., . . . Farrugia, G. (2014). Loss-of-function of the voltage-gated sodium channel NaV1.5 (channelopathies) in patients with irritable bowel syndrome. *Gastroenterology, 146*(7), 1659-1668. doi:10.1053/j.gastro.2014.02.054

Bian, X., Burda, J. E., Carrasquillo, M., & Galligan, J. J. (2009). Postnatal downregulation of inhibitory neuromuscular transmission to the longitudinal muscle of the guinea pig ileum. *Neurogastroenterol Motil, 21*(9), 969-977. doi:10.1111/j.1365-2982.2009.01296.x

Bian, X., Zhou, X., & Galligan, J. J. (2004). R-type calcium channels in myenteric neurons of guinea pig small intestine. *Am J Physiol Gastrointest Liver Physiol, 287*(1), G134-142. doi:10.1152/ajpgi.00532.2003

Bittencourt, A. F., Martins, J. R., Logullo, L., Shiroma, G., Horie, L., Ortolani, M. C., . . . Waitzberg, D. L. (2012). Constipation is more frequent than diarrhea in patients fed exclusively by enteral nutrition: results of an observational study. *Nutr Clin Pract, 27*(4), 533-539. doi:10.1177/0884533612449488

Bough, K. J., Wetherington, J., Hassel, B., Pare, J. F., Gawryluk, J. W., Greene, J. G., . . . Dingledine, R. J. (2006). Mitochondrial biogenesis in the anticonvulsant mechanism of the ketogenic diet. *Ann Neurol, 60*(2), 223-235. doi:10.1002/ana.20899

Bouza, A. A., Edokobi, N., Hodges, S. L., Pinsky, A. M., Offord, J., Piao, L., . . . Isom, L. L. (2021). Sodium channel beta1 subunits participate in regulated intramembrane proteolysis-excitation coupling. *JCI Insight*. doi:10.1172/jci.insight.141776

Braakman, H. M. H., & van Ingen, J. (2018). Can epilepsy be treated by antibiotics? *J Neurol, 265*(8), 1934-1936. doi:10.1007/s00415-018-8943-3

Brackenbury, W. J., Calhoun, J. D., Chen, C., Miyazaki, H., Nukina, N., Oyama, F., . . . Isom, L. L. (2010). Functional reciprocity between Na+ channel Nav1.6 and beta1 subunits in the coordinated regulation of excitability and neurite outgrowth. *Proc Natl Acad Sci U S A, 107*(5), 2283-2288. doi:10.1073/pnas.0909434107

Brackenbury, W. J., Davis, T. H., Chen, C., Slat, E. A., Detrow, M. J., Dickendesher, T. L., . . . Isom, L. L. (2008). Voltage-gated Na+ channel beta1 subunit-mediated neurite outgrowth requires Fyn kinase and contributes to postnatal CNS development in vivo. *J Neurosci, 28*(12), 3246-3256. doi:10.1523/JNEUROSCI.5446-07.2008

Brackenbury, W. J., & Isom, L. L. (2011). Na Channel beta Subunits: Overachievers of the Ion Channel Family. *Front Pharmacol, 2*, 53. doi:10.3389/fphar.2011.00053

Brackenbury, W. J., Yuan, Y., O'Malley, H. A., Parent, J. M., & Isom, L. L. (2013). Abnormal neuronal patterning occurs during early postnatal brain development of Scn1b-null mice and precedes hyperexcitability. *Proc Natl Acad Sci U S A, 110*(3), 1089-1094. doi:10.1073/pnas.1208767110

Bridgewater, M., Cunnane, T. C., & Brading, A. F. (1995). Characteristic features of inhibitory junction potentials evoked by single stimuli in the guinea-pig isolated taenia caeci. *J Physiol, 485 (Pt 1)*, 145-155. doi:10.1113/jphysiol.1995.sp020719

Brierley, S. M., Nichols, K., Grasby, D. J., & Waterman, S. A. (2001). Neural mechanisms underlying migrating motor complex formation in mouse isolated colon. *Br J Pharmacol, 132*(2), 507-517. doi:10.1038/sj.bjp.0703814

Brookes, S. J. (2001). Classes of enteric nerve cells in the guinea-pig small intestine. *Anat Rec, 262*(1), 58-70. doi:10.1002/1097-0185(20010101)262:1<58::AID-AR1011>3.0.CO;2-V

Brunklaus, A., Ellis, R., Reavey, E., Semsarian, C., & Zuberi, S. M. (2014). Genotype phenotype associations across the voltage-gated sodium channel family. *J Med Genet, 51*(10), 650-658. doi:10.1136/jmedgenet-2014-102608

Calhoun, J. D., & Isom, L. L. (2014). The role of non-pore-forming beta subunits in physiology and pathophysiology of voltage-gated sodium channels. *Handb Exp Pharmacol, 221*, 51-89. doi:10.1007/978-3-642-41588-3_4

Cannon, A., Kurklinsky, S., Guthrie, K. J., & Riegert-Johnson, D. L. (2016). Advanced Genetic Testing Comes to the Pain Clinic to Make a Diagnosis of Paroxysmal Extreme Pain Disorder. *Case Rep Neurol Med, 2016*, 9212369. doi:10.1155/2016/9212369

Caraballo, R. H., Cersosimo, R. O., Sakr, D., Cresta, A., Escobal, N., & Fejerman, N. (2005). Ketogenic diet in patients with Dravet syndrome. *Epilepsia, 46*(9), 1539-1544. doi:10.1111/j.1528-1167.2005.05705.x

Catterall, W. A. (2000). From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. *Neuron, 26*(1), 13-25. doi:10.1016/s0896-6273(00)81133-2

Catterall, W. A. (2012). Voltage-gated sodium channels at 60: structure, function and pathophysiology. *J Physiol, 590*(11), 2577-2589. doi:10.1113/jphysiol.2011.224204

Catterall, W. A., Goldin, A. L., & Waxman, S. G. (2005). International Union of Pharmacology. XLVII. Nomenclature and structure-function relationships of voltage-gated sodium channels. *Pharmacol Rev, 57*(4), 397-409. doi:10.1124/pr.57.4.4

Center, N. O. a. R. B. D. N. R. (2018). OI Issues: Constipation (NIH Publication No. 18-7915).

Chahine, M., Chatelier, A., Babich, O., & Krupp, J. J. (2008). Voltage-gated sodium channels in neurological disorders. *CNS Neurol Disord Drug Targets, 7*(2), 144-158. doi:10.2174/187152708784083830

Chen, C., Westenbroek, R. E., Xu, X., Edwards, C. A., Sorenson, D. R., Chen, Y., . . . Isom, L. L. (2004). Mice lacking sodium channel beta1 subunits display defects in neuronal excitability, sodium channel expression, and nodal architecture. *J Neurosci, 24*(16), 4030-4042. doi:10.1523/JNEUROSCI.4139-03.2004

Cheng H.M., M. K. K., Seluakumaran K. (2020). Motility: Peristalsis, Segmentation, Haustration and Mass Movement. In *Defining Physiology: Principles, Themes, Concepts* (Vol. 2): Springer, Cham.

Cheng H.M., M. K. K., Seluakumaran K. . (2020). Gastrointestinal Rhythmic Contractions Electrical Basis: Slow Wave Potential. In *Gastrointestinal Rhythmic Contractions Electrical Basis: Slow Wave Potential.* (Vol. 2, pp. 27-29): Springer, Cham.

Claes, L., Del-Favero, J., Ceulemans, B., Lagae, L., Van Broeckhoven, C., & De Jonghe, P. (2001). De novo mutations in the sodium-channel gene SCN1A cause severe myoclonic epilepsy of infancy. *Am J Hum Genet, 68*(6), 1327-1332. doi:10.1086/320609

Copel, C., Clerc, N., Osorio, N., Delmas, P., & Mazet, B. (2013). The Nav1.9 channel regulates colonic motility in mice. *Front Neurosci, 7*, 58. doi:10.3389/fnins.2013.00058

Cousins, H. M., Edwards, F. R., Hickey, H., Hill, C. E., & Hirst, G. D. (2003). Electrical coupling between the myenteric interstitial cells of Cajal and adjacent muscle layers in the guinea-pig gastric antrum. *J Physiol, 550*(Pt 3), 829-844. doi:10.1113/jphysiol.2003.042176

Cousins, H. M., Edwards, F. R., Hirst, G. D., & Wendt, I. R. (1993). Cholinergic neuromuscular transmission in the longitudinal muscle of the guinea-pig ileum. *J Physiol, 471*, 61-86. doi:10.1113/jphysiol.1993.sp019891

Craig, A. K., de Menezes, M. S., & Saneto, R. P. (2012). Dravet syndrome: patients with co-morbid SCN1A gene mutations and mitochondrial electron transport chain defects. *Seizure, 21*(1), 17-20. doi:10.1016/j.seizure.2011.08.010

Dabby, R., Sadeh, M., Broitman, Y., Yosovich, K., Dickman, R., & Leshinsky-Silver, E. (2016). Painful small fiber neuropathy with gastroparesis: A new phenotype with a novel mutation in the SCN10A gene. *J Clin Neurosci, 26*, 84-88. doi:10.1016/j.jocn.2015.05.071

Dahlin, M., Elfving, A., Ungerstedt, U., & Amark, P. (2005). The ketogenic diet influences the levels of excitatory and inhibitory amino acids in the CSF in children with refractory epilepsy. *Epilepsy Res, 64*(3), 115-125. doi:10.1016/j.eplepsyres.2005.03.008

Dahlin, M., & Prast-Nielsen, S. (2019). The gut microbiome and epilepsy. *EBioMedicine, 44*, 741-746. doi:10.1016/j.ebiom.2019.05.024

Davis, T. H., Chen, C., & Isom, L. L. (2004). Sodium channel beta1 subunits promote neurite outgrowth in cerebellar granule neurons. *J Biol Chem, 279*(49), 51424-51432. doi:10.1074/jbc.M410830200

de Kovel, C. G., Meisler, M. H., Brilstra, E. H., van Berkestijn, F. M., van 't Slot, R., van Lieshout, S., . . . Koeleman, B. P. (2014). Characterization of a de novo SCN8A mutation in a patient with epileptic encephalopathy. *Epilepsy Res, 108*(9), 1511-1518. doi:10.1016/j.eplepsyres.2014.08.020

de Vries, P., Soret, R., Suply, E., Heloury, Y., & Neunlist, M. (2010). Postnatal development of myenteric neurochemical phenotype and impact on neuromuscular transmission in the rat colon. *Am J Physiol Gastrointest Liver Physiol, 299*(2), G539-547. doi:10.1152/ajpgi.00092.2010

Debongnie, J. C., & Phillips, S. F. (1978). Capacity of the human colon to absorb fluid. *Gastroenterology, 74*(4), 698-703. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/631507

Dhar Malhotra, J., Chen, C., Rivolta, I., Abriel, H., Malhotra, R., Mattei, L. N., . . . Isom, L. L. (2001). Characterization of sodium channel alpha- and beta-subunits in rat and mouse cardiac myocytes. *Circulation, 103*(9), 1303-1310. doi:10.1161/01.cir.103.9.1303

Dickson, E. J., Heredia, D. J., McCann, C. J., Hennig, G. W., & Smith, T. K. (2010). The mechanisms underlying the generation of the colonic migrating motor complex in both wild-type and nNOS knockout mice. *Am J Physiol Gastrointest Liver Physiol, 298*(2), G222-232. doi:10.1152/ajpgi.00399.2009

Dieterich, W., Schink, M., & Zopf, Y. (2018). Microbiota in the Gastrointestinal Tract. *Med Sci (Basel), 6*(4). doi:10.3390/medsci6040116

Dravet, C. (2011). The core Dravet syndrome phenotype. *Epilepsia, 52 Suppl 2*, 3-9. doi:10.1111/j.1528-1167.2011.02994.x

Dukowicz, A. C., Lacy, B. E., & Levine, G. M. (2007). Small intestinal bacterial overgrowth: a comprehensive review. *Gastroenterol Hepatol (N Y), 3*(2), 112-122. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/21960820

Edokobi, N., & Isom, L. L. (2018). Voltage-Gated Sodium Channel beta1/beta1B Subunits Regulate Cardiac Physiology and Pathophysiology. *Front Physiol, 9*, 351. doi:10.3389/fphys.2018.00351

Eijkenboom, I., Sopacua, M., Hoeijmakers, J. G. J., de Greef, B. T. A., Lindsey, P., Almomani, R., . . . Gerrits, M. M. (2019). Yield of peripheral sodium channels gene screening in pure small fibre neuropathy. *J Neurol Neurosurg Psychiatry, 90*(3), 342-352. doi:10.1136/jnnp-2018-319042

Fein, A. J., Wright, M. A., Slat, E. A., Ribera, A. B., & Isom, L. L. (2008). scn1bb, a zebrafish ortholog of SCN1B expressed in excitable and nonexcitable cells, affects motor neuron axon morphology and touch sensitivity. *J Neurosci, 28*(47), 12510-12522. doi:10.1523/JNEUROSCI.4329-08.2008

Fertleman, C. R., Baker, M. D., Parker, K. A., Moffatt, S., Elmslie, F. V., Abrahamsen, B., . . . Rees, M. (2006). SCN9A mutations in paroxysmal extreme pain disorder: allelic variants underlie distinct channel defects and phenotypes. *Neuron, 52*(5), 767-774. doi:10.1016/j.neuron.2006.10.006

Fida, R., Lyster, D. J., Bywater, R. A., & Taylor, G. S. (1997). Colonic migrating motor complexes (CMMCs) in the isolated mouse colon. *Neurogastroenterol Motil, 9*(2), 99-107. doi:10.1046/j.1365-2982.1997.d01-25.x

Fiorotto, R., & Strazzabosco, M. (2019). Pathophysiology of Cystic Fibrosis Liver Disease: A Channelopathy Leading to Alterations in Innate Immunity and in Microbiota. *Cell Mol Gastroenterol Hepatol, 8*(2), 197-207. doi:10.1016/j.jcmgh.2019.04.013

Fish, E. M., & Burns, B. (2021). Physiology, Small Bowel. In *StatPearls*. Treasure Island (FL).

Foong, J. P., Tough, I. R., Cox, H. M., & Bornstein, J. C. (2014). Properties of cholinergic and non-cholinergic submucosal neurons along the mouse colon. *J Physiol, 592*(4), 777-793. doi:10.1113/jphysiol.2013.265686

Fung, C., & Vanden Berghe, P. (2020). Functional circuits and signal processing in the enteric nervous system. *Cell Mol Life Sci, 77*(22), 4505-4522. doi:10.1007/s00018-020-03543-6

Furness, J. B. (2000). Types of neurons in the enteric nervous system. *J Auton Nerv Syst, 81*(1-3), 87-96. doi:10.1016/s0165-1838(00)00127-2

Furness, J. B. (2007). Enteric nervous system. 2(10):4064.

Furness, J. B., Callaghan, B. P., Rivera, L. R., & Cho, H. J. (2014). The enteric nervous system and gastrointestinal innervation: integrated local and central control. *Adv Exp Med Biol, 817*, 39-71. doi:10.1007/978-1-4939-0897-4_3

Furness, J. B., & Costa, M. (1982). Neurons with 5-hydroxytryptamine-like immunoreactivity in the enteric nervous system: their projections in the guinea-pig small intestine. *Neuroscience, 7*(2), 341-349. doi:10.1016/0306-4522(82)90271-8

Furness, J. B., Costa, M., & Keast, J. R. (1984). Choline acetyltransferase- and peptide immunoreactivity of submucous neurons in the small intestine of the guinea-pig. *Cell Tissue Res, 237*(2), 329-336. doi:10.1007/BF00217152

Galley, J. D., Nelson, M. C., Yu, Z., Dowd, S. E., Walter, J., Kumar, P. S., . . . Bailey, M. T. (2014). Exposure to a social stressor disrupts the community structure of the

colonic mucosa-associated microbiota. *BMC Microbiol, 14*, 189. doi:10.1186/1471-2180-14-189
Galligan, J. J., LePard, K. J., Schneider, D. A., & Zhou, X. (2000). Multiple mechanisms of fast excitatory synaptic transmission in the enteric nervous system. *J Auton Nerv Syst, 81*(1-3), 97-103. doi:10.1016/s0165-1838(00)00130-2
Gardella, E., & Møller, R. S. (2019). Phenotypic and genetic spectrum of SCN8A-related disorders, treatment options, and outcomes. *Epilepsia, 60 Suppl 3*, S77-S85. doi:10.1111/epi.16319
Gay, S., Dupuis, D., Faivre, L., Masurel-Paulet, A., Labenne, M., Colombani, M., . . . Thauvin-Robinet, C. (2008). Severe neonatal non-dystrophic myotonia secondary to a novel mutation of the voltage-gated sodium channel (SCN4A) gene. *Am J Med Genet A, 146A*(3), 380-383. doi:10.1002/ajmg.a.32141
Gershon, M. D. (1999). The enteric nervous system: a second brain. *Hosp Pract (1995), 34*(7), 31-32, 35-38, 41-32 passim. doi:10.3810/hp.1999.07.153
Gershon, M. D. (2005). Nerves, reflexes, and the enteric nervous system: pathogenesis of the irritable bowel syndrome. *J Clin Gastroenterol, 39*(5 Suppl 3), S184-193. doi:10.1097/01.mcg.0000156403.37240.30
Giaroni, C., Knight, G. E., Ruan, H. Z., Glass, R., Bardini, M., Lecchini, S., . . . Burnstock, G. (2002). P2 receptors in the murine gastrointestinal tract. *Neuropharmacology, 43*(8), 1313-1323. doi:10.1016/s0028-3908(02)00294-0
Gomez-Eguilaz, M., Ramon-Trapero, J. L., Perez-Martinez, L., & Blanco, J. R. (2018). The beneficial effect of probiotics as a supplementary treatment in drug-resistant epilepsy: a pilot study. *Benef Microbes, 9*(6), 875-881. doi:10.3920/BM2018.0018
Gorard, D. A., Gomborone, J. E., Libby, G. W., & Farthing, M. J. (1996). Intestinal transit in anxiety and depression. *Gut, 39*(4), 551-555. doi:10.1136/gut.39.4.551
Greig, C. J., & Cowles, R. A. (2017). Muscarinic acetylcholine receptors participate in small intestinal mucosal homeostasis. *J Pediatr Surg, 52*(6), 1031-1034. doi:10.1016/j.jpedsurg.2017.03.037
Hall, A. B., Tolonen, A. C., & Xavier, R. J. (2017). Human genetic variation and the gut microbiome in disease. *Nat Rev Genet, 18*(11), 690-699. doi:10.1038/nrg.2017.63
Hamnett, R., Dershowitz, L. B., Sampathkumar, V., Wang, Z., De Andrade, V., Kasthuri, N., . . . Kaltschmidt, J. A. (2021). Regional cytoarchitecture of the adult and developing mouse enteric nervous system. *bioRxiv*. doi:https://doi.org/10.1101/2021.07.16.452735
Hansen, M. B. (2003). The enteric nervous system II: gastrointestinal functions. *Pharmacol Toxicol, 92*(6), 249-257. doi:10.1034/j.1600-0773.2003.920601.x
Hansen, M. B., & Skadhauge, E. (1997). Signal transduction pathways for serotonin as an intestinal secretagogue. *Comp Biochem Physiol A Physiol, 118*(2), 283-290. doi:10.1016/s0300-9629(97)00085-6
Hao, M. M., Bornstein, J. C., & Young, H. M. (2013). Development of myenteric cholinergic neurons in ChAT-Cre;R26R-YFP mice. *J Comp Neurol, 521*(14), 3358-3370. doi:10.1002/cne.23354
Hao, M. M., Lomax, A. E., McKeown, S. J., Reid, C. A., Young, H. M., & Bornstein, J. C. (2012). Early development of electrical excitability in the mouse enteric nervous

system. *J Neurosci, 32*(32), 10949-10960. doi:10.1523/JNEUROSCI.1426-12.2012
Hart, P. J., Overturf, K. E., Russell, S. N., Carl, A., Hume, J. R., Sanders, K. M., & Horowitz, B. (1993). Cloning and expression of a Kv1.2 class delayed rectifier K+ channel from canine colonic smooth muscle. *Proc Natl Acad Sci U S A, 90*(20), 9659-9663. doi:10.1073/pnas.90.20.9659
Hatton, W. J., Mason, H. S., Carl, A., Doherty, P., Latten, M. J., Kenyon, J. L., . . . Horowitz, B. (2001). Functional and molecular expression of a voltage-dependent K(+) channel (Kv1.1) in interstitial cells of Cajal. *J Physiol, 533*(Pt 2), 315-327. doi:10.1111/j.1469-7793.2001.0315a.x
Haufe, V., Camacho, J. A., Dumaine, R., Gunther, B., Bollensdorff, C., von Banchet, G. S., . . . Zimmer, T. (2005). Expression pattern of neuronal and skeletal muscle voltage-gated Na+ channels in the developing mouse heart. *J Physiol, 564*(Pt 3), 683-696. doi:10.1113/jphysiol.2004.079681
He, Z., Cui, B. T., Zhang, T., Li, P., Long, C. Y., Ji, G. Z., & Zhang, F. M. (2017). Fecal microbiota transplantation cured epilepsy in a case with Crohn's disease: The first report. *World J Gastroenterol, 23*(19), 3565-3568. doi:10.3748/wjg.v23.i19.3565
Hetz, S., Acikgoez, A., Moll, C., Jahnke, H. G., Robitzki, A. A., Metzger, R., & Metzger, M. (2014). Age-related gene expression analysis in enteric ganglia of human colon after laser microdissection. *Front Aging Neurosci, 6*, 276. doi:10.3389/fnagi.2014.00276
Hirst, C. S., Foong, J. P., Stamp, L. A., Fegan, E., Dent, S., Cooper, E. C., . . . McKeown, S. J. (2015). Ion channel expression in the developing enteric nervous system. *PLoS One, 10*(3), e0123436. doi:10.1371/journal.pone.0123436
Hirst, G. D., Holman, M. E., & Spence, I. (1974). Two types of neurones in the myenteric plexus of duodenum in the guinea-pig. *J Physiol, 236*(2), 303-326. doi:10.1113/jphysiol.1974.sp010436
Hisama, F. M., Dib-Hajj, S. D., & Waxman, S. G. (1993). SCN9A Neuropathic Pain Syndromes. In M. P. Adam, H. H. Ardinger, R. A. Pagon, S. E. Wallace, L. J. H. Bean, K. Stephens, & A. Amemiya (Eds.), *GeneReviews((R))*. Seattle (WA).
Holm, A. N., Rich, A., Miller, S. M., Strege, P., Ou, Y., Gibbons, S., . . . Farrugia, G. (2002). Sodium current in human jejunal circular smooth muscle cells. *Gastroenterology, 122*(1), 178-187. doi:10.1053/gast.2002.30346
Hooper, L. V., Wong, M. H., Thelin, A., Hansson, L., Falk, P. G., & Gordon, J. I. (2001). Molecular analysis of commensal host-microbial relationships in the intestine. *Science, 291*(5505), 881-884. doi:10.1126/science.291.5505.881
Horiguchi, K., & Komuro, T. (2000). Ultrastructural observations of fibroblast-like cells forming gap junctions in the W/W(nu) mouse small intestine. *J Auton Nerv Syst, 80*(3), 142-147. doi:10.1016/s0165-1838(00)00089-8
Howell, K. B., McMahon, J. M., Carvill, G. L., Tambunan, D., Mackay, M. T., Rodriguez-Casero, V., . . . Scheffer, I. E. (2015). SCN2A encephalopathy: A major cause of epilepsy of infancy with migrating focal seizures. *Neurology, 85*(11), 958-966. doi:10.1212/WNL.0000000000001926
Huizinga, J. D., Martz, S., Gil, V., Wang, X. Y., Jimenez, M., & Parsons, S. (2011). Two independent networks of interstitial cells of cajal work cooperatively with the

enteric nervous system to create colonic motor patterns. *Front Neurosci, 5*, 93. doi:10.3389/fnins.2011.00093
Huizinga, J. D., Thuneberg, L., Kluppel, M., Malysz, J., Mikkelsen, H. B., & Bernstein, A. (1995). W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. *Nature, 373*(6512), 347-349. doi:10.1038/373347a0
Hull, J. M., O'Malley, H. A., Chen, C., Yuan, Y., Denomme, N., Bouza, A. A., . . . Isom, L. L. (2020). Excitatory and inhibitory neuron defects in a mouse model of Scn1b-linked EIEE52. *Ann Clin Transl Neurol, 7*(11), 2137-2149. doi:10.1002/acn3.51205
Iino, S., & Horiguchi, K. (2006). Interstitial cells of cajal are involved in neurotransmission in the gastrointestinal tract. *Acta Histochem Cytochem, 39*(6), 145-153. doi:10.1267/ahc.06023
Isom, L. L. (2001). Sodium channel beta subunits: anything but auxiliary. *Neuroscientist, 7*(1), 42-54. doi:10.1177/107385840100700108
Isom, L. L. (2002). The role of sodium channels in cell adhesion. *Front Biosci, 7*, 12-23. doi:10.2741/isom
Isom, L. L., & Catterall, W. A. (1996). Na+ channel subunits and Ig domains. *Nature, 383*(6598), 307-308. doi:10.1038/383307b0
Isom, L. L., De Jongh, K. S., Patton, D. E., Reber, B. F., Offord, J., Charbonneau, H., . . . Catterall, W. A. (1992). Primary structure and functional expression of the beta 1 subunit of the rat brain sodium channel. *Science, 256*(5058), 839-842. doi:10.1126/science.1375395
Jahromi, S. R., Togha, M., Fesharaki, S. H., Najafi, M., Moghadam, N. B., Kheradmand, J. A., . . . Gorji, A. (2011). Gastrointestinal adverse effects of antiepileptic drugs in intractable epileptic patients. *Seizure, 20*(4), 343-346. doi:10.1016/j.seizure.2010.12.011
Jepps, T. A., Greenwood, I. A., Moffatt, J. D., Sanders, K. M., & Ohya, S. (2009). Molecular and functional characterization of Kv7 K+ channel in murine gastrointestinal smooth muscles. *Am J Physiol Gastrointest Liver Physiol, 297*(1), G107-115. doi:10.1152/ajpgi.00057.2009
Johanson, J. F., Sonnenberg, A., & Koch, T. R. (1989). Clinical epidemiology of chronic constipation. *J Clin Gastroenterol, 11*(5), 525-536. doi:10.1097/00004836-198910000-00008
Juge, N., Gray, J. A., Omote, H., Miyaji, T., Inoue, T., Hara, C., . . . Moriyama, Y. (2010). Metabolic control of vesicular glutamate transport and release. *Neuron, 68*(1), 99-112. doi:10.1016/j.neuron.2010.09.002
Kang, B. J., Chang, D. A., Mackay, D. D., West, G. H., Moreira, T. S., Takakura, A. C., . . . Stornetta, R. L. (2007). Central nervous system distribution of the transcription factor Phox2b in the adult rat. *J Comp Neurol, 503*(5), 627-641. doi:10.1002/cne.21409
Kaplan, D. I., Isom, L. L., & Petrou, S. (2016). Role of Sodium Channels in Epilepsy. *Cold Spring Harb Perspect Med, 6*(6). doi:10.1101/cshperspect.a022814
Kaufmann, S. G., Westenbroek, R. E., Maass, A. H., Lange, V., Renner, A., Wischmeyer, E., . . . Maier, S. K. G. (2013). Distribution and function of sodium channel subtypes in human atrial myocardium. *J Mol Cell Cardiol, 61*, 133-141. doi:10.1016/j.yjmcc.2013.05.006

Kazarinova-Noyes, K., Malhotra, J. D., McEwen, D. P., Mattei, L. N., Berglund, E. O., Ranscht, B., . . . Xiao, Z. C. (2001). Contactin associates with Na+ channels and increases their functional expression. *J Neurosci, 21*(19), 7517-7525. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/11567041

Kazen-Gillespie, K. A., Ragsdale, D. S., D'Andrea, M. R., Mattei, L. N., Rogers, K. E., & Isom, L. L. (2000). Cloning, localization, and functional expression of sodium channel beta1A subunits. *J Biol Chem, 275*(2), 1079-1088. doi:10.1074/jbc.275.2.1079

Kearney, J. (2013). Voltage-gated ion channel accessory subunits: sodium, potassium, or both? *Epilepsy Curr, 13*(1), 30-31. doi:10.5698/1535-7511-13.1.30

Keller, J., & Layer, P. (2014). The Pathophysiology of Malabsorption. *Viszeralmedizin, 30*(3), 150-154. doi:10.1159/000364794

Kim, M. S., Chung, N. G., Kang, M. R., Yoo, N. J., & Lee, S. H. (2011). Genetic and expressional alterations of CHD genes in gastric and colorectal cancers. *Histopathology, 58*(5), 660-668. doi:10.1111/j.1365-2559.2011.03819.x

Kim, Y. C., Koh, S. D., & Sanders, K. M. (2002). Voltage-dependent inward currents of interstitial cells of Cajal from murine colon and small intestine. *J Physiol, 541*(Pt 3), 797-810. doi:10.1113/jphysiol.2002.018796

Kito, Y., & Suzuki, H. (2003). Properties of pacemaker potentials recorded from myenteric interstitial cells of Cajal distributed in the mouse small intestine. *J Physiol, 553*(Pt 3), 803-818. doi:10.1113/jphysiol.2003.051334

Klein, S., Seidler, B., Kettenberger, A., Sibaev, A., Rohn, M., Feil, R., . . . Saur, D. (2013). Interstitial cells of Cajal integrate excitatory and inhibitory neurotransmission with intestinal slow-wave activity. *Nat Commun, 4*, 1630. doi:10.1038/ncomms2626

Kole, M. H., Ilschner, S. U., Kampa, B. M., Williams, S. R., Ruben, P. C., & Stuart, G. J. (2008). Action potential generation requires a high sodium channel density in the axon initial segment. *Nat Neurosci, 11*(2), 178-186. doi:10.1038/nn2040

Komada, M., & Soriano, P. (2002). [Beta]IV-spectrin regulates sodium channel clustering through ankyrin-G at axon initial segments and nodes of Ranvier. *J Cell Biol, 156*(2), 337-348. doi:10.1083/jcb.200110003

Kulkarni, S., Micci, M. A., Leser, J., Shin, C., Tang, S. C., Fu, Y. Y., . . . Pasricha, P. J. (2017). Adult enteric nervous system in health is maintained by a dynamic balance between neuronal apoptosis and neurogenesis. *Proc Natl Acad Sci U S A, 114*(18), E3709-E3718. doi:10.1073/pnas.1619406114

Kurahashi, M., Mutafova-Yambolieva, V., Koh, S. D., & Sanders, K. M. (2014). Platelet-derived growth factor receptor-alpha-positive cells and not smooth muscle cells mediate purinergic hyperpolarization in murine colonic muscles. *Am J Physiol Cell Physiol, 307*(6), C561-570. doi:10.1152/ajpcell.00080.2014

Lee, I. C., Chang, T. M., Liang, J. S., & Li, S. Y. (2019). KCNQ2 mutations in childhood nonlesional epilepsy: Variable phenotypes and a novel mutation in a case series. *Mol Genet Genomic Med, 7*(7), e00816. doi:10.1002/mgg3.816

Leipold, E., Liebmann, L., Korenke, G. C., Heinrich, T., Giesselmann, S., Baets, J., . . . Kurth, I. (2013). A de novo gain-of-function mutation in SCN11A causes loss of pain perception. *Nat Genet, 45*(11), 1399-1404. doi:10.1038/ng.2767

Li, Z. S., & Furness, J. B. (1998). Immunohistochemical localisation of cholinergic markers in putative intrinsic primary afferent neurons of the guinea-pig small intestine. *Cell Tissue Res, 294*(1), 35-43. doi:10.1007/s004410051154
Lin, X., O'Malley, H., Chen, C., Auerbach, D., Foster, M., Shekhar, A., . . . Delmar, M. (2015). Scn1b deletion leads to increased tetrodotoxin-sensitive sodium current, altered intracellular calcium homeostasis and arrhythmias in murine hearts. *J Physiol, 593*(6), 1389-1407. doi:10.1113/jphysiol.2014.277699
Linan-Rico, A., Ochoa-Cortes, F., Beyder, A., Soghomonyan, S., Zuleta-Alarcon, A., Coppola, V., & Christofi, F. L. (2016). Mechanosensory Signaling in Enterochromaffin Cells and 5-HT Release: Potential Implications for Gut Inflammation. *Front Neurosci, 10*, 564. doi:10.3389/fnins.2016.00564
Lindefeldt, M., Eng, A., Darban, H., Bjerkner, A., Zetterstrom, C. K., Allander, T., . . . Prast-Nielsen, S. (2019). The ketogenic diet influences taxonomic and functional composition of the gut microbiota in children with severe epilepsy. *NPJ Biofilms Microbiomes, 5*(1), 5. doi:10.1038/s41522-018-0073-2
Locke, G. R., 3rd, Ackerman, M. J., Zinsmeister, A. R., Thapa, P., & Farrugia, G. (2006). Gastrointestinal symptoms in families of patients with an SCN5A-encoded cardiac channelopathy: evidence of an intestinal channelopathy. *Am J Gastroenterol, 101*(6), 1299-1304. doi:10.1111/j.1572-0241.2006.00507.x
Lopez-Santiago, L. F., Brackenbury, W. J., Chen, C., & Isom, L. L. (2011). Na+ channel Scn1b gene regulates dorsal root ganglion nociceptor excitability in vivo. *J Biol Chem, 286*(26), 22913-22923. doi:10.1074/jbc.M111.242370
Lopez-Santiago, L. F., Meadows, L. S., Ernst, S. J., Chen, C., Malhotra, J. D., McEwen, D. P., . . . Isom, L. L. (2007). Sodium channel Scn1b null mice exhibit prolonged QT and RR intervals. *J Mol Cell Cardiol, 43*(5), 636-647. doi:10.1016/j.yjmcc.2007.07.062
Lum, G. R., Olson, C. A., & Hsiao, E. Y. (2020). Emerging roles for the intestinal microbiome in epilepsy. *Neurobiol Dis, 135*, 104576. doi:10.1016/j.nbd.2019.104576
Maggi, C. A., Catalioto, R. M., Criscuoli, M., Cucchi, P., Giuliani, S., Lecci, A., . . . Giachetti, A. (1997). Tachykinin receptors and intestinal motility. *Can J Physiol Pharmacol, 75*(6), 696-703. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/9276151
Maier, S. K., Westenbroek, R. E., McCormick, K. A., Curtis, R., Scheuer, T., & Catterall, W. A. (2004). Distinct subcellular localization of different sodium channel alpha and beta subunits in single ventricular myocytes from mouse heart. *Circulation, 109*(11), 1421-1427. doi:10.1161/01.CIR.0000121421.61896.24
Mane, N., Gil, V., Martinez-Cutillas, M., Clave, P., Gallego, D., & Jimenez, M. (2014). Differential functional role of purinergic and nitrergic inhibitory cotransmitters in human colonic relaxation. *Acta Physiol (Oxf), 212*(4), 293-305. doi:10.1111/apha.12408
Marini, C., Romoli, M., Parrini, E., Costa, C., Mei, D., Mari, F., . . . Guerrini, R. (2017). Clinical features and outcome of 6 new patients carrying de novo KCNB1 gene mutations. *Neurol Genet, 3*(6), e206. doi:10.1212/NXG.0000000000000206
Marionneau, C., Carrasquillo, Y., Norris, A. J., Townsend, R. R., Isom, L. L., Link, A. J., & Nerbonne, J. M. (2012). The sodium channel accessory subunit Navbeta1

regulates neuronal excitability through modulation of repolarizing voltage-gated K(+) channels. *J Neurosci, 32*(17), 5716-5727. doi:10.1523/JNEUROSCI.6450-11.2012
Masuda, M., Tomita, H., Okubo, K., & Miyasaka, K. (1994). Vagal efferent nerve-dependent inhibitory action of pancreatic polypeptide and peptide YY in conscious rats: comparison with somatostatin. *J Auton Nerv Syst, 50*(2), 131-138. doi:10.1016/0165-1838(94)90003-5
Mazurek, M. O., Vasa, R. A., Kalb, L. G., Kanne, S. M., Rosenberg, D., Keefer, A., . . . Lowery, L. A. (2013). Anxiety, sensory over-responsivity, and gastrointestinal problems in children with autism spectrum disorders. *J Abnorm Child Psychol, 41*(1), 165-176. doi:10.1007/s10802-012-9668-x
McElhanon, B. O., McCracken, C., Karpen, S., & Sharp, W. G. (2014). Gastrointestinal symptoms in autism spectrum disorder: a meta-analysis. *Pediatrics, 133*(5), 872-883. doi:10.1542/peds.2013-3995
McEwen, D. P., & Isom, L. L. (2004). Heterophilic interactions of sodium channel beta1 subunits with axonal and glial cell adhesion molecules. *J Biol Chem, 279*(50), 52744-52752. doi:10.1074/jbc.M405990200
McEwen, D. P., Meadows, L. S., Chen, C., Thyagarajan, V., & Isom, L. L. (2004). Sodium channel beta1 subunit-mediated modulation of Nav1.2 currents and cell surface density is dependent on interactions with contactin and ankyrin. *J Biol Chem, 279*(16), 16044-16049. doi:10.1074/jbc.M400856200
McKay, C. M., Ye, J., & Huizinga, J. D. (2006). Characterization of depolarization-evoked ERG K currents in interstitial cells of Cajal. *Neurogastroenterol Motil, 18*(4), 324-333. doi:10.1111/j.1365-2982.2006.00764.x
Meadows, L. S., Malhotra, J., Loukas, A., Thyagarajan, V., Kazen-Gillespie, K. A., Koopman, M. C., . . . Ragsdale, D. S. (2002). Functional and biochemical analysis of a sodium channel beta1 subunit mutation responsible for generalized epilepsy with febrile seizures plus type 1. *J Neurosci, 22*(24), 10699-10709. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/12486163
Moezi, L., Pirsalami, F., & Inaloo, S. (2015). Constipation enhances the propensity to seizure in pentylenetetrazole-induced seizure models of mice. *Epilepsy Behav, 44*, 200-206. doi:10.1016/j.yebeh.2015.01.013
Morais, L. H., Schreiber, H. L. t., & Mazmanian, S. K. (2021). The gut microbiota-brain axis in behaviour and brain disorders. *Nat Rev Microbiol, 19*(4), 241-255. doi:10.1038/s41579-020-00460-0
Morgan, K., Stevens, E. B., Shah, B., Cox, P. J., Dixon, A. K., Lee, K., . . . Jackson, A. P. (2000). beta 3: an additional auxiliary subunit of the voltage-sensitive sodium channel that modulates channel gating with distinct kinetics. *Proc Natl Acad Sci U S A, 97*(5), 2308-2313. doi:10.1073/pnas.030362197
Morris, C., Galuppi, B. E., & Rosenbaum, P. L. (2004). Reliability of family report for the Gross Motor Function Classification System. *Dev Med Child Neurol, 46*(7), 455-460. doi:10.1017/s0012162204000751
Murphy, M. C., & Fox, E. A. (2010). Mice deficient in brain-derived neurotrophic factor have altered development of gastric vagal sensory innervation. *J Comp Neurol, 518*(15), 2934-2951. doi:10.1002/cne.22372

Nakagawa, T., Misawa, H., Nakajima, Y., & Takaki, M. (2005). Absence of peristalsis in the ileum of W/W(V) mutant mice that are selectively deficient in myenteric interstitial cells of Cajal. *J Smooth Muscle Res, 41*(3), 141-151. doi:10.1540/jsmr.41.141

Nei, M., Ngo, L., Sirven, J. I., & Sperling, M. R. (2014). Ketogenic diet in adolescents and adults with epilepsy. *Seizure, 23*(6), 439-442. doi:10.1016/j.seizure.2014.02.015

Neshatian, L., Strege, P. R., Rhee, P. L., Kraichely, R. E., Mazzone, A., Bernard, C. E., . . . Farrugia, G. (2015). Ranolazine inhibits voltage-gated mechanosensitive sodium channels in human colon circular smooth muscle cells. *Am J Physiol Gastrointest Liver Physiol, 309*(6), G506-512. doi:10.1152/ajpgi.00051.2015

Niel, J. P. (1991). [Role of substance P in the nervous system control of digestive motility]. *Arch Int Physiol Biochim Biophys, 99*(5), A65-76. doi:10.3109/13813459109145918

Nordenbo, A. M., Andersen, J. R., & Andersen, J. T. (1996). Disturbances of ano-rectal function in multiple sclerosis. *J Neurol, 243*(6), 445-451. doi:10.1007/BF00900497

Norton, C., & Chelvanayagam, S. (2010). Bowel problems and coping strategies in people with multiple sclerosis. *Br J Nurs, 19*(4), 220, 221-226. doi:10.12968/bjon.2010.19.4.46783

O'Donnell, A. M., Coyle, D., & Puri, P. (2016). Decreased Nav1.9 channel expression in Hirschsprung's disease. *J Pediatr Surg, 51*(9), 1458-1461. doi:10.1016/j.jpedsurg.2016.05.007

O'Donnell, A. M., Nakamura, H., Tomuschat, C., Marayati, N. F., & Puri, P. (2019). Abnormal Scn1b and Fxyd1 gene expression in the pulled-through ganglionic colon may influence functional outcome in patients with Hirschsprung's disease. *Pediatr Surg Int, 35*(1), 9-14. doi:10.1007/s00383-018-4370-x

O'Malley, H. A., Hull, J. M., Clawson, B. C., Chen, C., Owens-Fiestan, G., Jameson, M. B., . . . Isom, L. L. (2019). Scn1b deletion in adult mice results in seizures and SUDEP. *Ann Clin Transl Neurol, 6*(6), 1121-1126. doi:10.1002/acn3.785

O'Malley, H. A., & Isom, L. L. (2015). Sodium channel beta subunits: emerging targets in channelopathies. *Annu Rev Physiol, 77*, 481-504. doi:10.1146/annurev-physiol-021014-071846

Ofei, S. Y., & Fuchs, G. J., 3rd. (2018). Constipation Burden in Children with Autism Spectrum Disorder: Emergency Department and Healthcare Use. *J Pediatr, 202*, 12-13. doi:10.1016/j.jpeds.2018.06.057

Ogiwara, I., Nakayama, T., Yamagata, T., Ohtani, H., Mazaki, E., Tsuchiya, S., . . . Yamakawa, K. (2012). A homozygous mutation of voltage-gated sodium channel beta(I) gene SCN1B in a patient with Dravet syndrome. *Epilepsia, 53*(12), e200-203. doi:10.1111/epi.12040

Ogobuiro, I., Gonzales, J., & Tuma, F. (2021). Physiology, Gastrointestinal. In *StatPearls*. Treasure Island (FL).

Oh, Y., Lee, Y. J., & Waxman, S. G. (1997). Regulation of Na+ channel beta 1 and beta 2 subunit mRNA levels in cultured rat astrocytes. *Neurosci Lett, 234*(2-3), 107-110. doi:10.1016/s0304-3940(97)00694-0

Ohkusa, T., Koido, S., Nishikawa, Y., & Sato, N. (2019). Gut Microbiota and Chronic Constipation: A Review and Update. *Front Med (Lausanne), 6*, 19. doi:10.3389/fmed.2019.00019

Olson, C. A., Vuong, H. E., Yano, J. M., Liang, Q. Y., Nusbaum, D. J., & Hsiao, E. Y. (2018). The Gut Microbiota Mediates the Anti-Seizure Effects of the Ketogenic Diet. *Cell, 174*(2), 497. doi:10.1016/j.cell.2018.06.051

Osorio, N., Korogod, S., & Delmas, P. (2014). Specialized functions of Nav1.5 and Nav1.9 channels in electrogenesis of myenteric neurons in intact mouse ganglia. *J Neurosci, 34*(15), 5233-5244. doi:10.1523/JNEUROSCI.0057-14.2014

Osteen, J. D., Herzig, V., Gilchrist, J., Emrick, J. J., Zhang, C., Wang, X., . . . Julius, D. (2016). Selective spider toxins reveal a role for the Nav1.1 channel in mechanical pain. *Nature, 534*(7608), 494-499. doi:10.1038/nature17976

Ou, Y., Gibbons, S. J., Miller, S. M., Strege, P. R., Rich, A., Distad, M. A., . . . Farrugia, G. (2002). SCN5A is expressed in human jejunal circular smooth muscle cells. *Neurogastroenterol Motil, 14*(5), 477-486. doi:10.1046/j.1365-2982.2002.00348.x

Overturf, K. E., Russell, S. N., Carl, A., Vogalis, F., Hart, P. J., Hume, J. R., . . . Horowitz, B. (1994). Cloning and characterization of a Kv1.5 delayed rectifier K+ channel from vascular and visceral smooth muscles. *Am J Physiol, 267*(5 Pt 1), C1231-1238. doi:10.1152/ajpcell.1994.267.5.C1231

Padilla, F., Couble, M. L., Coste, B., Maingret, F., Clerc, N., Crest, M., . . . Delmas, P. (2007). Expression and localization of the Nav1.9 sodium channel in enteric neurons and in trigeminal sensory endings: implication for intestinal reflex function and orofacial pain. *Mol Cell Neurosci, 35*(1), 138-152. doi:10.1016/j.mcn.2007.02.008

Parsons, S. P., & Huizinga, J. D. (2010). Transient outward potassium current in ICC. *Am J Physiol Gastrointest Liver Physiol, 298*(3), G456-466. doi:10.1152/ajpgi.00340.2009

Patel, B. A., Dai, X., Burda, J. E., Zhao, H., Swain, G. M., Galligan, J. J., & Bian, X. (2010). Inhibitory neuromuscular transmission to ileal longitudinal muscle predominates in neonatal guinea pigs. *Neurogastroenterol Motil, 22*(8), 909-918, e236-907. doi:10.1111/j.1365-2982.2010.01508.x

Patino, G. A., Brackenbury, W. J., Bao, Y., Lopez-Santiago, L. F., O'Malley, H. A., Chen, C., . . . Isom, L. L. (2011). Voltage-gated Na+ channel beta1B: a secreted cell adhesion molecule involved in human epilepsy. *J Neurosci, 31*(41), 14577-14591. doi:10.1523/JNEUROSCI.0361-11.2011

Patino, G. A., Claes, L. R., Lopez-Santiago, L. F., Slat, E. A., Dondeti, R. S., Chen, C., . . . Isom, L. L. (2009). A functional null mutation of SCN1B in a patient with Dravet syndrome. *J Neurosci, 29*(34), 10764-10778. doi:10.1523/JNEUROSCI.2475-09.2009

Patino, G. A., & Isom, L. L. (2010). Electrophysiology and beyond: multiple roles of Na+ channel beta subunits in development and disease. *Neurosci Lett, 486*(2), 53-59. doi:10.1016/j.neulet.2010.06.050

Peiris, M., Hockley, J. R., Reed, D. E., Smith, E. S. J., Bulmer, D. C., & Blackshaw, L. A. (2017). Peripheral KV7 channels regulate visceral sensory function in mouse and human colon. *Mol Pain, 13*, 1744806917709371. doi:10.1177/1744806917709371

Peng, A., Qiu, X., Lai, W., Li, W., Zhang, L., Zhu, X., . . . Chen, L. (2018). Altered composition of the gut microbiome in patients with drug-resistant epilepsy. *Epilepsy Res, 147*, 102-107. doi:10.1016/j.eplepsyres.2018.09.013

Pereira, D. B., & Chao, M. V. (2007). The tyrosine kinase Fyn determines the localization of TrkB receptors in lipid rafts. *J Neurosci, 27*(18), 4859-4869. doi:10.1523/JNEUROSCI.4587-06.2007

Perez-Medina, A. L., & Galligan, J. J. (2019). Optogenetic analysis of neuromuscular transmission in the colon of ChAT-ChR2-YFP BAC transgenic mice. *Am J Physiol Gastrointest Liver Physiol, 317*(5), G569-G579. doi:10.1152/ajpgi.00089.2019

Peri, L. E., Sanders, K. M., & Mutafova-Yambolieva, V. N. (2013). Differential expression of genes related to purinergic signaling in smooth muscle cells, PDGFRalpha-positive cells, and interstitial cells of Cajal in the murine colon. *Neurogastroenterol Motil, 25*(9), e609-620. doi:10.1111/nmo.12174

Personal_Communication (2019 & 2020). [De-identified OMIM DEE52 patient growth data and symptomology was collected via personal communication
with Annapurna Poduri, MD, MPH (May 6, 2020 - Boston Children's
Hospital); Christelle Achkar, MD (March 27, 2019 - Boston Children's
Hospital); Fowzan S. Alkuraya, MD; Mais O. Hashem (April 7, 2019 - King Faisal Specialist
Hospital and Research Center); Katalin Štěrbová, MD (March 20, 2019 - Charles University
and Motol Hospital); Alec Aeby, MD, PhD (April 8, 2019 - Hôpital Universitaire Des Enfants
Reine Fabiola (HUDERF)).].

Pham, T. D., Gershon, M. D., & Rothman, T. P. (1991). Time of origin of neurons in the murine enteric nervous system: sequence in relation to phenotype. *J Comp Neurol, 314*(4), 789-798. doi:10.1002/cne.903140411

Phillips, S. F., & Giller, J. (1973). The contribution of the colon to electrolyte and water conservation in man. *J Lab Clin Med, 81*(5), 733-746. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/4698660

Powley, T. L., & Phillips, R. J. (2011). Vagal intramuscular array afferents form complexes with interstitial cells of Cajal in gastrointestinal smooth muscle: analogues of muscle spindle organs? *Neuroscience, 186*, 188-200. doi:10.1016/j.neuroscience.2011.04.036

Qu, Z. D., Thacker, M., Castelucci, P., Bagyanszki, M., Epstein, M. L., & Furness, J. B. (2008). Immunohistochemical analysis of neuron types in the mouse small intestine. *Cell Tissue Res, 334*(2), 147-161. doi:10.1007/s00441-008-0684-7

Quigley, E. M. (2011). Microflora modulation of motility. *J Neurogastroenterol Motil, 17*(2), 140-147. doi:10.5056/jnm.2011.17.2.140

Ramadan, W., Patel, N., Anazi, S., Kentab, A. Y., Bashiri, F. A., Hamad, M. H., . . . Alkuraya, F. S. (2017). Confirming the recessive inheritance of SCN1B mutations in developmental epileptic encephalopathy. *Clin Genet, 92*(3), 327-331. doi:10.1111/cge.12999

Rao, M., & Gershon, M. D. (2016). The bowel and beyond: the enteric nervous system in neurological disorders. *Nat Rev Gastroenterol Hepatol, 13*(9), 517-528. doi:10.1038/nrgastro.2016.107

Rao, M., & Gershon, M. D. (2018). Enteric nervous system development: what could possibly go wrong? *Nat Rev Neurosci, 19*(9), 552-565. doi:10.1038/s41583-018-0041-0

Rao, S. S., & Go, J. T. (2010). Update on the management of constipation in the elderly: new treatment options. *Clin Interv Aging, 5*, 163-171. doi:10.2147/cia.s8100

Reed, D. E., & Vanner, S. J. (2001). Converging and diverging cholinergic inputs from submucosal neurons amplify activity of secretomotor neurons in guinea-pig ileal submucosa. *Neuroscience, 107*(4), 685-696. doi:10.1016/s0306-4522(01)00392-x

Reed, D. E., & Vanner, S. J. (2003). Long vasodilator reflexes projecting through the myenteric plexus in guinea-pig ileum. *J Physiol, 553*(Pt 3), 911-924. doi:10.1113/jphysiol.2003.053140

Reid, C. A., Leaw, B., Richards, K. L., Richardson, R., Wimmer, V., Yu, C., . . . Petrou, S. (2014). Reduced dendritic arborization and hyperexcitability of pyramidal neurons in a Scn1b-based model of Dravet syndrome. *Brain, 137*(Pt 6), 1701-1715. doi:10.1093/brain/awu077

Ren, J., & Bertrand, P. P. (2008). Purinergic receptors and synaptic transmission in enteric neurons. *Purinergic Signal, 4*(3), 255-266. doi:10.1007/s11302-007-9088-5

Roberts, R. R., Murphy, J. F., Young, H. M., & Bornstein, J. C. (2007). Development of colonic motility in the neonatal mouse-studies using spatiotemporal maps. *Am J Physiol Gastrointest Liver Physiol, 292*(3), G930-938. doi:10.1152/ajpgi.00444.2006

Rodriguez-Tapia, E., Perez-Medina, A., Bian, X., & Galligan, J. J. (2016). Upregulation of L-type calcium channels in colonic inhibitory motoneurons of P/Q-type calcium channel-deficient mice. *Am J Physiol Gastrointest Liver Physiol, 311*(4), G763-G774. doi:10.1152/ajpgi.00263.2016

Rodriguez-Tapia, E. S., Naidoo, V., DeVries, M., Perez-Medina, A., & Galligan, J. J. (2017). R-Type Ca(2+) channels couple to inhibitory neurotransmission to the longitudinal muscle in the guinea-pig ileum. *Exp Physiol, 102*(3), 299-313. doi:10.1113/EP086027

Rogawski, M. A., Loscher, W., & Rho, J. M. (2016). Mechanisms of Action of Antiseizure Drugs and the Ketogenic Diet. *Cold Spring Harb Perspect Med, 6*(5). doi:10.1101/cshperspect.a022780

Rowland, I., Gibson, G., Heinken, A., Scott, K., Swann, J., Thiele, I., & Tuohy, K. (2018). Gut microbiota functions: metabolism of nutrients and other food components. *Eur J Nutr, 57*(1), 1-24. doi:10.1007/s00394-017-1445-8

Rugiero, F., Gola, M., Kunze, W. A., Reynaud, J. C., Furness, J. B., & Clerc, N. (2002). Analysis of whole-cell currents by patch clamp of guinea-pig myenteric neurones in intact ganglia. *J Physiol, 538*(Pt 2), 447-463. doi:10.1113/jphysiol.2001.013051

Sage, D., Salin, P., Alcaraz, G., Castets, F., Giraud, P., Crest, M., . . . Clerc, N. (2007). Na(v)1.7 and Na(v)1.3 are the only tetrodotoxin-sensitive sodium channels

expressed by the adult guinea pig enteric nervous system. *J Comp Neurol, 504*(4), 363-378. doi:10.1002/cne.21450
Saito, Y. A., Strege, P. R., Tester, D. J., Locke, G. R., 3rd, Talley, N. J., Bernard, C. E., . . . Farrugia, G. (2009). Sodium channel mutation in irritable bowel syndrome: evidence for an ion channelopathy. *Am J Physiol Gastrointest Liver Physiol, 296*(2), G211-218. doi:10.1152/ajpgi.90571.2008
Sanders, K. M. (2019). Spontaneous Electrical Activity and Rhythmicity in Gastrointestinal Smooth Muscles. *Adv Exp Med Biol, 1124*, 3-46. doi:10.1007/978-981-13-5895-1_1
Sanders, K. M., Koh, S. D., Ro, S., & Ward, S. M. (2012). Regulation of gastrointestinal motility--insights from smooth muscle biology. *Nat Rev Gastroenterol Hepatol, 9*(11), 633-645. doi:10.1038/nrgastro.2012.168
Sanders, K. M., & Ward, S. M. (1992). Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. *Am J Physiol, 262*(3 Pt 1), G379-392. doi:10.1152/ajpgi.1992.262.3.G379
Sarna, S. K., Condon, R., & Cowles, V. (1984). Colonic migrating and nonmigrating motor complexes in dogs. *Am J Physiol, 246*(4 Pt 1), G355-360. doi:10.1152/ajpgi.1984.246.4.G355
Sasselli, V., Boesmans, W., Vanden Berghe, P., Tissir, F., Goffinet, A. M., & Pachnis, V. (2013). Planar cell polarity genes control the connectivity of enteric neurons. *J Clin Invest, 123*(4), 1763-1772. doi:10.1172/JCI66759
Schmidt, U., Weigert, M., Broaddus, C., & Myers, G. (2018). *Cell Detection with Star-convex Polygons*. Paper presented at the International Conference on Medical Image Computing and Computer-Assisted Intervention, Granada, Spain.
Schneider, S., Wright, C. M., & Heuckeroth, R. O. (2019). Unexpected Roles for the Second Brain: Enteric Nervous System as Master Regulator of Bowel Function. *Annu Rev Physiol, 81*, 235-259. doi:10.1146/annurev-physiol-021317-121515
Seifi, M., Brown, J. F., Mills, J., Bhandari, P., Belelli, D., Lambert, J. J., . . . Swinny, J. D. (2014). Molecular and functional diversity of GABA-A receptors in the enteric nervous system of the mouse colon. *J Neurosci, 34*(31), 10361-10378. doi:10.1523/JNEUROSCI.0441-14.2014
Sharma, S., & Tripathi, M. (2013). Ketogenic diet in epileptic encephalopathies. *Epilepsy Res Treat, 2013*, 652052. doi:10.1155/2013/652052
Shuttleworth, C. W., & Smith, T. K. (1999). Action potential-dependent calcium transients in myenteric S neurons of the guinea-pig ileum. *Neuroscience, 92*(2), 751-762. doi:10.1016/s0306-4522(99)00012-3
Simkin, D., & Bendahhou, S. (2011). Skeletal muscle na channel disorders. *Front Pharmacol, 2*, 63. doi:10.3389/fphar.2011.00063
Smith, T. K., Kang, S. H., & Vanden Berghe, P. (2003). Calcium channels in enteric neurons. *Curr Opin Pharmacol, 3*(6), 588-593. doi:10.1016/j.coph.2003.10.001
Smith, T. K., Spencer, N. J., Hennig, G. W., & Dickson, E. J. (2007). Recent advances in enteric neurobiology: mechanosensitive interneurons. *Neurogastroenterol Motil, 19*(11), 869-878. doi:10.1111/j.1365-2982.2007.01019.x
Spencer, N. J. (2001). Control of migrating motor activity in the colon. *Curr Opin Pharmacol, 1*(6), 604-610. doi:10.1016/s1471-4892(01)00103-5

Spencer, N. J., Dickson, E. J., Hennig, G. W., & Smith, T. K. (2006). Sensory elements within the circular muscle are essential for mechanotransduction of ongoing peristaltic reflex activity in guinea-pig distal colon. *J Physiol, 576*(Pt 2), 519-531. doi:10.1113/jphysiol.2006.109561

Spencer, N. J., Dinning, P. G., Brookes, S. J., & Costa, M. (2016). Insights into the mechanisms underlying colonic motor patterns. *J Physiol, 594*(15), 4099-4116. doi:10.1113/JP271919

Spencer, N. J., Hennig, G. W., & Smith, T. K. (2003). Stretch-activated neuronal pathways to longitudinal and circular muscle in guinea pig distal colon. *Am J Physiol Gastrointest Liver Physiol, 284*(2), G231-241. doi:10.1152/ajpgi.00291.2002

Spinelli, E., & Blackford, R. (2018). Gut Microbiota, the Ketogenic Diet and Epilepsy. *Pediatr Neurol Briefs, 32*, 10. doi:10.15844/pedneurbriefs-32-10

Srivastava, S., Desai, S., Cohen, J., Smith-Hicks, C., Baranano, K., Fatemi, A., & Naidu, S. (2018). Monogenic disorders that mimic the phenotype of Rett syndrome. *Neurogenetics, 19*(1), 41-47. doi:10.1007/s10048-017-0535-3

Srivastava, S., & Sahin, M. (2017). Autism spectrum disorder and epileptic encephalopathy: common causes, many questions. *J Neurodev Disord, 9*, 23. doi:10.1186/s11689-017-9202-0

Stanislawski, M. A., Dabelea, D., Wagner, B. D., Sontag, M. K., Lozupone, C. A., & Eggesbo, M. (2017). Pre-pregnancy weight, gestational weight gain, and the gut microbiota of mothers and their infants. *Microbiome, 5*(1), 113. doi:10.1186/s40168-017-0332-0

Strege, P. R., Holm, A. N., Rich, A., Miller, S. M., Ou, Y., Sarr, M. G., & Farrugia, G. (2003). Cytoskeletal modulation of sodium current in human jejunal circular smooth muscle cells. *Am J Physiol Cell Physiol, 284*(1), C60-66. doi:10.1152/ajpcell.00532.2001

Strege, P. R., Knutson, K., Eggers, S. J., Li, J. H., Wang, F., Linden, D., . . . Beyder, A. (2017). Sodium channel NaV1.3 is important for enterochromaffin cell excitability and serotonin release. *Sci Rep, 7*(1), 15650. doi:10.1038/s41598-017-15834-3

Strege, P. R., Mazzone, A., Bernard, C. E., Neshatian, L., Gibbons, S. J., Saito, Y. A., . . . Farrugia, G. (2018). Irritable bowel syndrome patients have SCN5A channelopathies that lead to decreased NaV1.5 current and mechanosensitivity. *Am J Physiol Gastrointest Liver Physiol, 314*(4), G494-G503. doi:10.1152/ajpgi.00016.2017

Strege, P. R., Mazzone, A., Kraichely, R. E., Sha, L., Holm, A. N., Ou, Y., . . . Farrugia, G. (2007). Species dependent expression of intestinal smooth muscle mechanosensitive sodium channels. *Neurogastroenterol Motil, 19*(2), 135-143. doi:10.1111/j.1365-2982.2006.00844.x

Strege, P. R., Ou, Y., Sha, L., Rich, A., Gibbons, S. J., Szurszewski, J. H., . . . Farrugia, G. (2003). Sodium current in human intestinal interstitial cells of Cajal. *Am J Physiol Gastrointest Liver Physiol, 285*(6), G1111-1121. doi:10.1152/ajpgi.00152.2003

Sun, C., Chen, L., & Shen, Z. (2019). Mechanisms of gastrointestinal microflora on drug metabolism in clinical practice. *Saudi Pharm J, 27*(8), 1146-1156. doi:10.1016/j.jsps.2019.09.011

Suzuki, H., Ward, S. M., Bayguinov, Y. R., Edwards, F. R., & Hirst, G. D. (2003). Involvement of intramuscular interstitial cells in nitrergic inhibition in the mouse gastric antrum. *J Physiol, 546*(Pt 3), 751-763. doi:10.1113/jphysiol.2002.033365

Thomsen, L., Robinson, T. L., Lee, J. C., Farraway, L. A., Hughes, M. J., Andrews, D. W., & Huizinga, J. D. (1998). Interstitial cells of Cajal generate a rhythmic pacemaker current. *Nat Med, 4*(7), 848-851. doi:10.1038/nm0798-848

Thorneloe, K. S., & Nelson, M. T. (2005). Ion channels in smooth muscle: regulators of intracellular calcium and contractility. *Can J Physiol Pharmacol, 83*(3), 215-242. doi:10.1139/y05-016

Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., . . . Gordon, J. I. (2009). A core gut microbiome in obese and lean twins. *Nature, 457*(7228), 480-484. doi:10.1038/nature07540

Udit, S., & Gautron, L. (2013). Molecular anatomy of the gut-brain axis revealed with transgenic technologies: implications in metabolic research. *Front Neurosci, 7*, 134. doi:10.3389/fnins.2013.00134

van den Berg, M. M., Benninga, M. A., & Di Lorenzo, C. (2006). Epidemiology of childhood constipation: a systematic review. *Am J Gastroenterol, 101*(10), 2401-2409. doi:10.1111/j.1572-0241.2006.00771.x

Vezyroglou, K., & Cross, J. H. (2016). Targeted Treatment in Childhood Epilepsy Syndromes. *Curr Treat Options Neurol, 18*(6), 29. doi:10.1007/s11940-016-0407-4

Villas, N., Meskis, M. A., & Goodliffe, S. (2017). Dravet syndrome: Characteristics, comorbidities, and caregiver concerns. *Epilepsy Behav, 74*, 81-86. doi:10.1016/j.yebeh.2017.06.031

Vogalis, F., Harvey, J. R., Lohman, R. J., & Furness, J. B. (2002). Action potential afterdepolarization mediated by a Ca2+-activated cation conductance in myenteric AH neurons. *Neuroscience, 115*(2), 375-393. doi:10.1016/s0306-4522(02)00410-4

Wang, H. X., & Wang, Y. P. (2016). Gut Microbiota-brain Axis. *Chin Med J (Engl), 129*(19), 2373-2380. doi:10.4103/0366-6999.190667

Wang, J., Ou, S. W., & Wang, Y. J. (2017). Distribution and function of voltage-gated sodium channels in the nervous system. *Channels (Austin), 11*(6), 534-554. doi:10.1080/19336950.2017.1380758

Wang, X. Y., Paterson, C., & Huizinga, J. D. (2003). Cholinergic and nitrergic innervation of ICC-DMP and ICC-IM in the human small intestine. *Neurogastroenterol Motil, 15*(5), 531-543. doi:10.1046/j.1365-2982.2003.00429.x

Wang, Y. Q., Fang, Z. X., Zhang, Y. W., Xie, L. L., & Jiang, L. (2020). Efficacy of the ketogenic diet in patients with Dravet syndrome: A meta-analysis. *Seizure, 81*, 36-42. doi:10.1016/j.seizure.2020.07.011

Wang, Z. J., Bergqvist, C., Hunter, J. V., Jin, D., Wang, D. J., Wehrli, S., & Zimmerman, R. A. (2003). In vivo measurement of brain metabolites using two-dimensional double-quantum MR spectroscopy--exploration of GABA levels in a ketogenic diet. *Magn Reson Med, 49*(4), 615-619. doi:10.1002/mrm.10429

Waxenbaum, J. A., Reddy, V., & Varacallo, M. (2021). Anatomy, Autonomic Nervous System. In *StatPearls*. Treasure Island (FL).

Wessler, I., Dooley, D. J., Werhand, J., & Schlemmer, F. (1990). Differential effects of calcium channel antagonists (omega-conotoxin GVIA, nifedipine, verapamil) on the electrically-evoked release of [3H]acetylcholine from the myenteric plexus, phrenic nerve and neocortex of rats. *Naunyn Schmiedebergs Arch Pharmacol, 341*(4), 288-294. doi:10.1007/BF00180653

Winge, K., Rasmussen, D., & Werdelin, L. M. (2003). Constipation in neurological diseases. *J Neurol Neurosurg Psychiatry, 74*(1), 13-19. doi:10.1136/jnnp.74.1.13

Wirrell, E. C., Laux, L., Donner, E., Jette, N., Knupp, K., Meskis, M. A., . . . Berg, A. T. (2017). Optimizing the Diagnosis and Management of Dravet Syndrome: Recommendations From a North American Consensus Panel. *Pediatr Neurol, 68*, 18-34 e13. doi:10.1016/j.pediatrneurol.2017.01.025

Wittmeyer, V., Merrot, T., & Mazet, B. (2010). Tonic inhibition of human small intestinal motility by nitric oxide in children but not in adults. *Neurogastroenterol Motil, 22*(10), 1078-e1282. doi:10.1111/j.1365-2982.2010.01532.x

Wood, J. D. (2004). Peristalsis. In L. R. Johnson (Ed.), *Encyclopedia of Gastroenterology* (pp. 164-165): Elsevier.

Woods, C. G., Babiker, M. O., Horrocks, I., Tolmie, J., & Kurth, I. (2015). The phenotype of congenital insensitivity to pain due to the NaV1.9 variant p.L811P. *Eur J Hum Genet, 23*(5), 561-563. doi:10.1038/ejhg.2014.166

Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y. Y., Keilbaugh, S. A., . . . Lewis, J. D. (2011). Linking long-term dietary patterns with gut microbial enterotypes. *Science, 334*(6052), 105-108. doi:10.1126/science.1208344

Wu, Y., Proch, K. L., Teran, F. A., Lechtenberg, R. J., Kothari, H., & Richerson, G. B. (2019). Chemosensitivity of Phox2b-expressing retrotrapezoid neurons is mediated in part by input from 5-HT neurons. *J Physiol, 597*(10), 2741-2766. doi:10.1113/JP277052

Xie, G., Zhou, Q., Qiu, C. Z., Dai, W. K., Wang, H. P., Li, Y. H., . . . Wang, W. J. (2017). Ketogenic diet poses a significant effect on imbalanced gut microbiota in infants with refractory epilepsy. *World J Gastroenterol, 23*(33), 6164-6171. doi:10.3748/wjg.v23.i33.6164

Xue, J., Askwith, C., Javed, N. H., & Cooke, H. J. (2007). Autonomic nervous system and secretion across the intestinal mucosal surface. *Auton Neurosci, 133*(1), 55-63. doi:10.1016/j.autneu.2007.02.001

Yu, F. H., Westenbroek, R. E., Silos-Santiago, I., McCormick, K. A., Lawson, D., Ge, P., . . . Curtis, R. (2003). Sodium channel beta4, a new disulfide-linked auxiliary subunit with similarity to beta2. *J Neurosci, 23*(20), 7577-7585. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/12930796

Yuan, Y., O'Malley, H. A., Smaldino, M. A., Bouza, A. A., Hull, J. M., & Isom, L. L. (2019). Delayed maturation of GABAergic signaling in the Scn1a and Scn1b mouse models of Dravet Syndrome. *Sci Rep, 9*(1), 6210. doi:10.1038/s41598-019-42191-0

Yudkoff, M., Daikhin, Y., Nissim, I., Horyn, O., Lazarow, A., Luhovyy, B., . . . Nissim, I. (2005). Response of brain amino acid metabolism to ketosis. *Neurochem Int, 47*(1-2), 119-128. doi:10.1016/j.neuint.2005.04.014

Zaman, T., Helbig, I., Bozovic, I. B., DeBrosse, S. D., Bergqvist, A. C., Wallis, K., . . . Goldberg, E. M. (2018). Mutations in SCN3A cause early infantile epileptic encephalopathy. *Ann Neurol, 83*(4), 703-717. doi:10.1002/ana.25188

Zheng, H., Park, K. S., Koh, S. D., & Sanders, K. M. (2014). Expression and function of a T-type Ca2+ conductance in interstitial cells of Cajal of the murine small intestine. *Am J Physiol Cell Physiol, 306*(7), C705-713. doi:10.1152/ajpcell.00390.2013

Zhu, X., Han, Y., Du, J., Liu, R., Jin, K., & Yi, W. (2017). Microbiota-gut-brain axis and the central nervous system. *Oncotarget, 8*(32), 53829-53838. doi:10.18632/oncotarget.17754

Zhu, Y. F., Wang, X. Y., Lowie, B. J., Parsons, S., White, L., Kunze, W., . . . Huizinga, J. D. (2014). Enteric sensory neurons communicate with interstitial cells of Cajal to affect pacemaker activity in the small intestine. *Pflugers Arch, 466*(7), 1467-1475. doi:10.1007/s00424-013-1374-1

Ziobro, J., Eschbach, K., Sullivan, J. E., & Knupp, K. G. (2018). Current Treatment Strategies and Future Treatment Options for Dravet Syndrome. *Curr Treat Options Neurol, 20*(12), 52. doi:10.1007/s11940-018-0537-y